

ECOIMMUNOLOGY OF THE ASIAN LONGHORNED BEETLE,  
*ANOPLOPHORA GLABRIPENNIS*

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by

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Insect-pathogen interactions can be influenced by many factors including host life history, behavior and abiotic factors, such as chemical exposure. Additionally, exposure to pathogens can have transgenerational implications and influence the susceptibility of offspring. Understanding the outcomes of these interactions can also have implications for developing effective control strategies for insect pests. Asian longhorned beetles, *Anoplophora glabripennis*, are invasive wood borers which have been introduced into North America and Europe and the entomopathogenic fungus *Metarhizium brunneum* is being developed for their control. Studies were conducted to determine how sexual maturity, insect age, mating status (unmated vs mated), host thermoregulatory behavior, and prior maternal pathogen exposure influence *A. glabripennis* susceptibility to *M. brunneum* and ability to suppress a fungal infection, even across a generation. Additionally, potential mechanisms of synergy between *M. brunneum* and the neonicotinoid pesticide imidacloprid were investigated. Beetles did not exhibit thermoregulatory behavior although it would have been beneficial. Prior maternal pathogen exposure enhanced offspring survival but whether offspring survival was enhanced depended on the pathogen used to expose mothers and whether or not the pathogen was living or dead. Beetle susceptibility to *A. glabripennis* was

also influenced by the age and mating status of beetles. We found that old beetles were not more susceptible than younger beetles and only found a cost of mating for mature but not old beetles. Finally we found that starvation may be a potential mechanism for the synergy between a pesticide (imidacloprid) and the fungal pathogen *M. brunneum*. These findings provide insight into insect-pathogen interactions and ecoimmunology in beetles.

## BIOGRAPHICAL SKETCH

Joanna Fisher was born to Greg and Janice Fisher in Oregon in 1987. She fell in love with insects while raising painted lady butterflies and exploring her family's backyard. She was homeschooled until she completed high school after which she attended Portland Community College and graduated with her Associates of Science in 2008. She transferred to Oregon State University where she took entomology courses and conducted an undergraduate thesis research project with Dr. Denny Bruck studying the entomopathogenic fungi associated with the rhizosphere of perennial herbs, shrubs and coniferous trees. She graduated in 2010 with her Bachelor's of Science.

In 2010 Joanna joined Dr. Ann Hajek's invertebrate pathology lab at Cornell University as a Ph.D. student. Her research focused on understanding the interactions between the Asian longhorned beetle, *Anoplophora glabripennis*, and the fungal pathogen *M. brunneum*. Following graduation, Joanna intends to continue to work with fungal entomopathogens studying insect-pathogen interactions and biological control.

Dedicated to my parents, Janice and Greg, and my siblings, Hannah, Jessica and Jonathan. You guys are great!

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My parents Greg and Janice have always encouraged and supported my love of science and insects, without them I highly doubt I would have pursued a career in science. From a young age my mom cultivated in me a love of learning and encouraged me to pursue my own unique interests. She even allowed me to raise numerous types of insects and even a spider in her kitchen despite the fact that she really didn't like insects (other than butterflies and praying mantises). When my dad discovered my interest in insects he taught himself biology and learned how to pin insects. My siblings Hanna, Jessica and Jonathan have been wonderful supporters and compatriots and I am so grateful to have you in my life. Thank you guys, you are amazing!

My other early mentor was MaryJo Andersen, a zookeeper and manager of the endangered butterfly lab at the Oregon zoo. She took a chance on me in high school by allowing me to volunteer at the lab even though I was very young to be an intern. I fell in love with insect pathology while working and conducting research as an undergraduate in Dr. Denny Bruck's lab at the USDA-ARS in Corvallis, OR. I am incredibly grateful for his patient mentoring and guidance.

I would like to thank my advisor, Dr. Ann Hajek for allowing me to pursue my interests in graduate school. Through Ann's mentorship I developed into an independent scientist for which I will always be grateful. I especially thank her for helping me troubleshoot problems with my methods and experiments. She provided excellent comments on numerous manuscript drafts including hundreds of references and thanks to her my writing skills have drastically improved. I look forward to future opportunities to collaborate.

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# Chapter 1: Introduction to ecoimmunology in beetles

## 1. Introduction

The immunity of insects is shaped by their ecology and evolution. The field of ecoimmunology brings together the fields of ecology and immunology to study variations in immunity and to examine why these variations exist (Martin et al., 2011; Schmid-Hempel, 2005). Ecoimmunology examines variation in individual immunity due to physiology, life history, biotic and abiotic factors, sociality and population genetics. An idea central to ecoimmunology is the assumption that immunity is costly and organisms must optimize their immune response to maximize their overall fitness (Schmid-Hempel, 2005; Sheldon and Verhulst, 1996). Costs of immunity include energetic costs, loss of fitness if immunity trades-off with other tasks and self-harm resulting from mounting an immune response.

Immunity is not only costly and potentially dangerous but it is also only one aspect of the response organisms can mount against pathogen and parasite infection and will therefore vary depending on the ecology and evolution of the parasite and host (Martin et al., 2011). Organisms can maximize their fitness upon infection using behavioral defenses (grooming, pathogen avoidance, etc.), resisting the pathogen (removing the pathogen), tolerating the pathogen (reducing the impact of the pathogen), acquiring immunity or altering their reproduction (Ayres and Schneider, 2012; Boots et

al., 2009; Martin et al., 2011).

Insects provide tractable model organisms with which to study ecoimmunology and much work has already been conducted on insect ecoimmunology. In this review I will focus specifically on ecoimmunology in beetles and examine how immunity can trade-off with other physiological processes such as mating and age and how it is shaped by sexual selection. Additionally, I will examine how the biotic community, specifically prior pathogen exposure, as well as abiotic factors influence immunity in beetles. There are other aspects of beetles' ecology and evolution which can lead to variation in immunity that will not be addressed in this literature review, including population genetics (Zhong et al., 2013) and social immunity (Cotter et al., 2013).

## 2. Physiology and life-history

### 2.1 Gender differences in immunocompetence

Immunity can be influenced by gender and evolutionary theory predicts that males and females should invest differently in immunity (Rolff, 2002; Stoehr and Kokko, 2006). Specifically, males will maximize their immediate fitness by investing in reproductive opportunities at the expense of their immunity while females will maximize their long-term fitness and therefore their longevity because of their greater investment in reproduction. Evidence supporting these predictions has been found in Coleoptera; female *Tenebrio molitor* [Tenebrionidae] invest more in immunity (had higher phenoloxidase levels), live longer (Hangartner et al., 2013) and have higher survival upon exposure to the fungal pathogen *Beauveria bassiana* than males (Valtonen et al., 2010).

Additionally in *T. molitor*, enhanced immunocompetence caused males to lose sperm precedence over time while it did not impact female fitness (Drnevich et al., 2002).

Female burying beetles, *Nicrophorus vespilloides* [Silphidae], which engage in cooperative brood care, invested more in immunity than their male partners and had higher antimicrobial activity in their anal exudates (used to protect offspring food resources from microbes) (Cotter and Kilner, 2010).

The ‘immunocompetency hypothesis’ which was first postulated by Hamilton and Zuk (1982), states that for vertebrates males secondary sexual traits provide honest signals of immunocompetence because testosterone, which is needed to produce them, is immunosuppressive. This hypothesis has now been extended to insects and Rantala et al. (2003), found that Juvenile Hormone (JH), which is involved in reproduction and development, may provide a possible mechanism supporting the immunocompetence hypothesis. When injected with JH *T. molitor* were more attractive to females and JH suppressed some aspects of the males’ immune response. Juvenile Hormone may also mediate mating-related immunosuppression in both male and female *T. molitor* (Rolff and Siva-Jothy, 2002).

Despite these predictions phenoloxidase (PO) activity was positively correlated with horn length in male horned beetles, *Euoniticellus intermedius* [Scarabaeidae], which use their horns to fight off other males (Pomfret and Knell, 2006). Furthermore, in male rhinoceros beetles, *Trypoxylus dichotomus* [Scarabaeidae], there was no evidence of a cost of maintaining a weapon and large horns did not weaken the beetle’s immune response or impair their survival in the field (McCullough and Emlen, 2013). While Demuth et al. (2012), found that larger horns may be costly to maintain since adult male

broad-horned flour beetles, *Gnatocerus cornutus* [Tenebrionidae], which were infected as larvae with the tapeworm parasite, *Hymenolepis diminuta*, had shorter horn length. It is also possible that beetles trade off horn length for body length as infection with *H. diminuta* did not reduce body size which was found to be more important in determining the outcome of sexual conflicts than horn size. It could be that among beetles with horns, the development of larger horns does not suppress male immunity as larger horns resulted in more robust immune responses and did not impair survival in the field.

Contrary to evolutionary theory, males of some beetle species invest more in immunity and live longer than females. Female *Anoplophora glabripennis* [Cerambycidae] are more susceptible to the fungal pathogen *Metarhizium brunneum*, do not have a greater immune response to a foreign challenge and have reduced longevity compared to males (Chapter 5). The encapsulation response of female horned beetles, *E. intermedius*, was the same as males though females did have higher PO activity suggesting that some aspects of a female's immune response may be up-regulated compared to males (Pomfret and Knell, 2006).

## 2.2 Sexual selection and sexual conflict

Sexual selection is predicted to influence male and female investment in immunity and is a likely mechanism of sexual dimorphism since males and females optimize their immunity differently (Rolff, 2002; Stoehr and Kokko, 2006). Under greater sexual selection male immunocompetence should decrease while in situations with little or no sexual selection male immunocompetence may be higher than or similar to that of females, as there would be little pressure for males to trade off investing in



immunity to maximize their fitness (Stoehr and Koko, 2006). Female *Tribolium castaneum* [Tenebrionidae] which evolved under high sexual selection (polyandry) had higher levels of PO activity compared to males, while there was no difference between the PO levels among genders that evolved under low sexual selection (monogamy) (Hangartner et al., 2015). However, this contrasts with results of earlier work by Hangartner et al. (2013) who reported finding no evidence for a genetic trade-off between investment in reproduction and immunity in three different *T. castaneum* lines under different sexual selection intensities (female-biased, male-biased or equal gender ratio) (Hangartner et al., 2013).

Sexual selection can also impact female immunity and females may decrease their investment in immunity to maximize their short-term fitness. In *Callosobruchus maculatus* [Chrysomelidae] both male and female beetles had decreased antimicrobial peptide responses under high sexual selection (male-biased lines) compared to low sexual selection (female-biased lines) (van Lieshout et al., 2014). These findings suggest that among beetles, sexual selection doesn't always lead to sexual dimorphisms in immunity and it is likely that other mechanisms besides sexual selection are involved.

### 2.3 Maturation and age

As beetles develop, mature, and age, their immunocompetence can change (Daukste et al., 2012; Khan et al., 2016; Maehara and Kanzaki, 2014). The effect of age on beetle immunocompetence is complex and varies depending on beetle species. For example, only young vs. old larvae of *T. castaneum* were susceptible to the microsporidian pathogen *Nosema whitei* (Blaser and Schmid-Hempel, 2005). Within the

adult lifespan, susceptibility can change as beetles mature. *Monochamus alternatus* [Cerambycidae], take 3 weeks to reach maturity after emergence and their resistance to *B. bassiana* increased from beetle emergence to day 14 (Maehara and Kanzaki, 2014). Male, but not female, *A. glabripennis* are also more susceptible prior to sexual maturation compared to older beetles (Fisher and Hajek, 2016).

One argument for age having an adverse effect on immunocompetence is that as animals age, the immune system breaks down and has a diminished capacity to properly react and combat a pathogen challenge (immunosenescence) (Stanley, 2012). A decline in immunocompetence with increasing age has been found for *M. alternatus* (Maehara and Kanzaki, 2014), *T. castaneum* (Khan et al., 2016) and male *T. molitor* beetles (Daukšte et al., 2012). However the impact of age on immunity can be complex and some aspects of insect immune responses may be increased even as insect susceptibility increases with age. Older *T. castaneum* beetles were more susceptible to *Bacillus thuringiensis* but their antibacterial and PO activity increased with age (Khan et al., 2016). The up-regulation of these immune responses may not be beneficial as the innate immune system has been shown to cause damage to host tissues in *T. molitor* (Sadd and Siva-Jothy, 2006) and may be the result of a loss of gene regulation associated with senescence (Stanley, 2012).

## 2.4 Interactions between gender, age and reproduction

The immunocompetence of individual beetles can be influenced by multiple interacting factors which can have complex and sometimes unpredictable effects. In *T. castaneum*, gender was found to influence immunosenescence and only female beetles

showed age-related declines in their external defenses (Khan et al., 2016). In *A. glabripennis*, only young male but not young female beetles were more susceptible to a pathogen challenge compared to older male and female beetles respectively (Fisher and Hajek, 2016). In *T. castaneum* whether or not mating status reduced post-infection survival was dependent on the gender of the beetles and only the survival of females was reduced (Khan et al., 2016), while in *A. glabripennis*, mating only increased susceptibility post-infection in mature but not old beetles (Fisher and Hajek, 2016). Finally, gender, mating status, and age were all found to interact and impact external defenses in *T. castaneum* (Khan et al., 2016).

### 3. Prior pathogen exposure

Individual immune competence can be shaped by prior pathogen exposure. In invertebrates, exposure to an immune challenge (a pathogen or pathogen-derived factor) can enhance the immune response to a subsequent challenge, a phenomenon known as immune priming (Kurtz and Armitage, 2006). Among beetles immune priming can occur within a life stage (Krams et al., 2013; Milutinovic et al., 2013; Moret and Siva-Jothy, 2003; Roth et al., 2009) and persist throughout development (ontogenic priming) (Thomas and Rudolf, 2010). Prior pathogen exposure can also enhance the immunocompetence of offspring in the next generation, an effect called trans-generational immune priming (TGIP) (Eggert et al., 2014; Fisher and Hajek, 2015; Knorr et al., 2015; Roth et al., 2010; Tate and Graham, 2015; Zanchi et al., 2011).

Priming in coleopteran insects has been found in response to immune elicitors (lipopolysaccharides (LPS) and nylon filaments) (Milutinovic et al., 2013), bacteria

(Fisher and Hajek, 2015; Roth et al., 2009), fungal pathogens (Fisher and Hajek, 2015) and gregarine parasites (Tate and Graham, 2015). In some cases, prior pathogen exposure protected beetles only against the same pathogen or pathogen strain previously experienced (Roth et al., 2009) while in other cases the primed response was found to provide non-specific protection (Fisher and Hajek, 2015). The resistance of *T. castaneum* beetles which were both primed and challenged with the same bacterial strain was significantly enhanced compared to priming and challenge with different bacteria strains, while in *A. glabripennis* maternal exposure to a bacterial pathogen provided non-specific protection during offspring exposure to a fungal pathogen (Fisher and Hajek, 2015).

Priming can also be influenced by the biotic community. *T. castaneum* larvae which were raised germ free exhibited a reduced primed response and died faster upon pathogen challenge compared to beetles which had their normal microbiota (Futo et al., 2016). Co-infection can also influence the outcome of TGIP (Tate and Graham, 2015). Priming of *T. castaneum* with *B. thuringiensis* enhanced offspring survival, however if parents were co-infected with *B. thuringiensis* and a gregarine gut parasite then offspring survival against *B. thuringiensis* infection was severely reduced. The virulence of the priming pathogen can influence the primed response and *T. castaneum* beetles had enhanced survival to *B. thuringiensis* when primed with media used to grow a pathogenic *B. thuringiensis* strain but not when they were exposed to media used to grow a non-pathogenic strain of *B. thuringiensis* (Milutinovic et al., 2013).

Priming can be costly and has been found to increase larval development time, reduce growth and pupal mass, increase mortality at metamorphosis and lower fecundity (Milutinovic et al., 2013; Roth et al., 2009; Roth and Kurtz, 2008; Thomas and Rudolf,

2010). However it is unclear if priming is always costly. The cost of priming has been found to vary based on what pathogen was used to prime beetles (Roth and Kurtz, 2008), whether TGIP occurred as a result of maternal or paternal pathogen exposure (Roth et al., 2009) and how the insect was exposed to the pathogen (through media vs. injection of heat-killed bacteria) (Milutinovic et al., 2013; Roth and Kurtz, 2008). Clearly further research needs to be conducted to investigate the costs of priming and under what circumstances priming is costly.

## 4. Abiotic Factors

### 4.1 Temperature

Temperature can influence insect immunity and can be used by insects to combat pathogen infections (Eggert et al., 2015; Hutchison and Erskine, 1981). Little is known about the impact of temperature on beetle immunity. In a few cases, exposure of beetles to high temperatures has been found to enhance their survival and immunity (Catalán et al., 2012b; Fisher and Hajek, 2014). *M. brunneum*-infected *A. glabripennis* exposed to 34°C for five hours a day lived significantly longer than beetles held at or below 31°C (Fisher and Hajek, 2014). Exposure to high temperatures increased the PO and antibacterial activity of both control and LPS-injected *T. molitor* beetles (Catalán et al., 2012b). Temperature exposure can also have transgenerational effects on beetle immunity. The survival of offspring exposed to *B. thuringiensis* was significantly enhanced upon parental exposure to a cold shock but not a heat shock (Eggert et al., 2015). There is also evidence that some beetles can alter their behavior and exhibit behavioral fever in response to pathogen exposure (Catalán et al., 2012b; McClain et al.,

1988).

Behavioral fever is the process by which insects behaviorally elevate their body temperature above their normal preferred temperature in response to parasitism or a pathogen challenge (Hutchison and Erskine, 1981). Behavioral fever has been found in Namib desert beetles (*Onymacris plana* [Tenebrionidae]) (McClain et al., 1988) and *T. molitor* larvae (Catalán et al., 2012a) which increased their preferred body temperature when injected with LPS. Behavioral fever was also examined in *A. glabripennis* and although exposure to high temperatures significantly delayed mortality compared to infected beetles, adult *A. glabripennis* did not exhibit behavioral fever when infected (Fisher and Hajek, 2014). It is possible that beetles were not physiologically adapted to fever or that fevering may have been too costly. When *T. molitor* were kept at 30°C and challenged with LPS they had a higher standard metabolic rate than unchallenged larvae held at 30°C while at lower temperatures there was no difference in the standard metabolic rate in challenged vs. unchallenged larvae (Catalán et al., 2012b).

## 4.2 Pesticide exposure and immunity

Exposure to pesticides can increase beetle susceptibility to pathogens. Pesticides have been found to increase beetle susceptibility to nematodes (Koppenhöfer et al., 2000) and fungal pathogens (Brito et al., 2008; Furlong and Groden, 2001; Quintela and McCoy, 1998; Russell et al., 2010). Pesticides may increase insect susceptibility by interfering with the encapsulation and melanization immune responses of beetles. Both an organophosphorus and a neonicotinoid pesticide have been found to reduce the melanization and encapsulation response of insects exposed to fungal pathogens

(Dubovskiy et al., 2013; Chapter 5). Some pesticides, particularly neonicotinoids, interfere with the ability of beetles to feed which can make them more susceptible to pathogens (Furlong and Groden, 2001; Chapter 5).

Pesticides, such as neonicotinoids, which cause continuous stimulation of the insect nervous system (Nauen and Denholm, 2005) can alter behavior and behavioral immune responses (Koppenhofer et al., 2000; Quintela and McCoy, 1998). Exposure of *Popillia japonica* [Scarabaeidae], larvae to the neonicotinoid insecticide imidacloprid, reduced the mobility and grooming behavior of the larvae making them more susceptible to nematode attack (Koppenhofer et al., 2000). Imidacloprid was also found to reduce the movement of *Diaprepes abbreviates* [Curculionidae], larvae in soil thus increasing their susceptibility to fungal infection (Quintela and McCoy, 1998).

### 4.3 Food quality

Food quality and availability can significantly impact beetle immunity and susceptibility to pathogens (Furlong and Groden, 2001; Siva-Jothy and Thompson, 2002). Starvation has been found to increase the susceptibility of *A. glabripennis* and *Leptinotarsa decemlineata* [Chrysomelidae] to fungal pathogens (Chapter 5; Furlong and Groden, 2001). Starvation and access to only low-nutrient food reduced PO activity in *T. molitor* (Rantala et al., 2003; Siva-Jothy and Thompson, 2002) but had no impact on beetles' encapsulation response (Chapter 5; Rantala et al., 2003; Siva-Jothy and Thompson., 2002). In the field, xylophagous *Tomicus piniperda* [Curculionidae] collected from high-quality food sources (stressed trees) were more resistant to *Beauveria bassiana* infection than beetles collected from low-quality food sources (healthy trees)

(Krams et al., 2012). While starvation and low-quality food can increase susceptibility of beetles, access to nutrients can alleviate the effects of parasitism, *T. molitor* infected with the tapeworm parasite *H. diminuta* did not show a decrease in reproduction if allowed to feed on a complementary nutrient sources (Ponton et al., 2011).

## 5. Conclusions and future directions

The ecology, physiology, life history, and biotic as well as abiotic factors can influence the immunity and susceptibility of beetles to pathogens. The gender of the beetle, whether or not it has mated and the age of the beetle can have complex interactions impacting host immunity and susceptibility. Beetles are part of a larger biotic community and prior pathogen exposure has been found to increase the immune response and survival of beetles both within a generation and across a generation. Beetle immunity can also be shaped by abiotic factors such as temperature and pesticide exposure which can alter their immune responses, and increase their susceptibility to pathogens. Beetles can use temperature to their advantage which may allow them to overcome or reduce the effects of a pathogen challenge.

Many aspects of ecoimmunology have not been examined in Coleoptera to date and research examining the influence of host microbiota on immunity as well as studies addressing how parasites and pathogens can manipulate their hosts' immunity are lacking. Additionally, little work has been conducted examining how abiotic factors other than temperature and pesticides influence immunity. The immunology of beetles in field populations is not influenced by a single factor and more studies examining the combined effects of host life history, physiology as well as biotic and abiotic factors on host



immunity are needed to understand how beetles modulate immunity to maximize their fitness. Future research should also focus on developing genomic tools which can be used to examine the mechanisms of host-pathogen interactions in the context of ecoimmunology. This field would also benefit from the development of consistent, standardized methods of measuring the immune response as well as standardized methods to detect costs and tradeoffs which can be used across a wide range of beetle species as well as other insects.

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Chapter 2. Thermoregulatory behavior and fungal infection of  
*Anoplophora glabripennis* (Coleoptera: Cerambycidae)

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## Abstract

Asian longhorned beetles, *Anoplophora glabripennis* (Motschulsky), are invasive wood borers that are native to China and Korea but have been introduced to North America and Europe. These beetles have great potential to negatively impact economic and environmental interests in hardwood and urban forests if they become established. The entomopathogenic fungus *Metarhizium brunneum* Petch (previously *M. anisopliae* (Metsch.) Sorokin) is under development for control of *A. glabripennis*. Some insect species eliminate pathogens or delay disease progression through thermoregulation. Because Asian longhorned beetles had been observed occupying sunlit areas of the tree canopy, we hypothesized that behavioral fevering could be used to delay mortality of fungal-infected beetles. *Metarhizium brunneum* cultures incubated at 34°C for 5 h/d grew significantly slower compared to cultures incubated at lower temperatures. Holding *M. brunneum*-infected *A. glabripennis* at 34°C for 5 h/d significantly delayed mortality by 2 d compared to infected beetles held at  $\leq 31^{\circ}\text{C}$ . Adult *A. glabripennis* did not exhibit behavioral fever when infected. Uninfected males, when provided with food and both uninfected males and females when deprived of food, slightly increased their preferred temperature over time. When held at 15°C prior to being placed into temperature gradients, uninfected beetles did not increase their temperatures above ambient. Results demonstrate that *M. brunneum*-infected *A. glabripennis* do not exhibit behaviors necessary to elevate their body temperatures enough to combat *M. brunneum* infections through thermoregulation.

Key Words: behavioral fever, thermoregulation, *Metarhizium brunneum*, Asian

longhorned beetle, biological control

# 1. Introduction

Some insects display altered behaviors in response to pathogen or parasite infection, either as a result of manipulation by the pathogen or macro-parasite or as a host defensive strategy (Shapiro-Ilan et al., 2012). Behavioral fever, “the process by which infected or parasitized insects behaviorally elevate their preferred or selected body temperature above normothermic levels” (Hutchison and Erskine, 1981), is one defensive strategy employed by some insects to combat infection or parasitism.

Behavioral fever can be elicited through infection with macro-parasites (including tachinids), bacteria or fungi/microsporidia in multiple insect species in several different orders, including Orthoptera (Adamo, 1998; Boorstein and Ewald, 1987; Bunday et al., 2003; Louis et al., 1986), Lepidoptera (Karbon, 1998), Diptera (Watson et al., 1993), Hymenoptera (Starks et al., 2000), Blattodea (Bronstein and Conner, 1984) and Coleoptera (Catalán et al., 2012; McClain et al., 1998). Many orthopterans exhibit behavioral fever in response to fungal pathogens. Senegalese grasshoppers (*Oedaleus senegalensis* (Krauss)) (Blanford et al., 1998; Blanford and Thomas, 1999), desert locusts (*Schistocerca gregaria* (Forskål)) (Bunday et al., 2003; Elliot et al., 2002), and migratory locusts (*Locusta migratoria migratorioides* (R. and F.)) (Ouedraogo et al., 2004) exhibited behavioral fever when exposed to *Metarhizium acridum* (Driver and Milner J.F. Bisch., Rehner and Humber (sometimes referred to as *M. flavoviride* Gams and Roszypal) while migratory grasshoppers, *Melanoplus sanguinipes* (F.), increased their fecundity when infected with the microsporidian *Tubulinosema acridophagus* (Henry) through fevering (Boorstein and Ewald, 1987). While house crickets (*Acheta domesticus* (L.)) (Adamo, 1998) and *Gryllus bimaculatus* De Geer exhibited behavioral fever when

infected with the bacterium *Rickettsiella grylli* (Vago and Martoja) (Louis et al., 1986).

Behavioral fever has also been observed in lepidopteran larvae (*Platyrepia virginalis* Boisduval) parasitized by a tachinid (*Thelaira americana* Brooks) (Karban, 1998). Dipteran house flies (*Musca domestica* L.) exhibited behavioral fever when infected with *Entomophthora muscae* (Cohn) Fresenius (Watson et al., 1993), or *Beauveria bassiana* (Bals.-Criv.) Vuillemin (Anderson et al., 2013a) but were only able to overcome the pathogenic effects of *E. muscae*. The honey bee (*Apis mellifera* L.) increased the temperature of the brood comb when brood were infected with chalkbrood (*Ascosphaera apis* (Maassen and Claussen) Olive and Spilator) (Starks et al., 2000), and honey bees infected with *Nosema ceranae* (Fries et al., 1996) preferred higher temperatures (Campbell et al., 2010). Behavioral fever is also exhibited by the Madagascar cockroach (*Gromphadorhina portentosa* Schaum) when infected with bacteria or injected with bacterial lipopolysaccharides (LPS) (Bronstein and Conner, 1984).

Namib desert beetles (*Onymacris plana* Perringuey) (McClain et al., 1998) and yellow mealworms (*Tenebrio molitor* L.) larvae (Catalán et al., 2012) exhibit behavioral fever when injected with LPS and behavioral thermoregulation occurs among scarabs (Chappell, 1984; Heinrich and McClain, 1986), silphids (Merrick and Smith, 2004) and carabids (Pineda and Kondratieff, 2003), many of which bask in the sun after a cold night, prior to flight or activity. It has also been suggested that the cerambycid *Stenurella melanura* Linné behaviorally thermoregulates through basking (Larsson and Krustvall, 1990).

Asian longhorned beetles, *Anoplophora glabripennis* (Motschulsky), are wood

borers native to China and Korea that were initially detected in North America in 1996. Breeding populations have been found in five eastern states in the US, one province in Canada, and six European countries (Fera, 2012; Haack et al., 2010; Loomans et al., 2013; Ohio Department of Agriculture, 2011). The entomopathogenic fungus *Metarhizium brunneum* Petch (formerly *Metarhizium anisopliae* (Metsch.) Sorokin) is pathogenic to *A. glabripennis* (Dubois et al., 2008). Non-woven fiber bands impregnated with *M. brunneum* and wrapped around trunks and branches of threatened trees have shown promise in controlling these insects. Adult beetles in China died significantly faster and laid fewer eggs in plots treated with these fungal bands compared with beetles from untreated plots (Hajek et al., 2006). In numerous systems host thermoregulatory behavior significantly decreases efficacy of fungal biological control products in the field (Thomas and Blanford, 2003). Therefore, we investigated whether *A. glabripennis* uses thermoregulatory behaviors in response to *M. brunneum* infection. Such behavior could mitigate the effects of infection through increasing survival to improve fitness.

In China, *A. glabripennis* has been observed basking in the sunlight at the tops of tree canopies in the morning (T. Poland, personal communication). Among other reasons, thermoregulation to achieve a preferred body temperature may be one mechanism that would explain this behavior. Given the observation that this beetle may thermoregulate, and given that other Coleoptera have been observed to exhibit behavioral fever in response to LPS (Catalán et al., 2012; McClain et al, 1998), we examined whether *A. glabripennis* exhibits behavioral fever in response to infection with *M. brunneum*. We hypothesized that *M. brunneum* growth in vitro would be inhibited when exposed to increasing temperatures and that infected *A. glabripennis* would live longer when kept at



warmer temperatures. We also hypothesized that *A. glabripennis* adults would exhibit behavioral fever in response to *M. brunneum* infections when placed in temperature gradient cages. Additionally, we expected healthy beetles when placed in temperature gradients to exhibit thermoregulatory behavior after cold exposure.

## 2. Materials and methods

### 2.1 Beetle colony

*Anoplophora glabripennis* were reared and tested under quarantine and under USDA APHIS permit at Cornell University's Sarkaria Arthropod Research Laboratory. The colony was started in 1998 with early-instar larvae collected from Langfang City, Hebei and Yongjing, Gansu, China and beetles from the Chicago infestation were added in 1999 (Dubois et al., 2002). Striped maple twigs (*Acer pensylvanicum* L.) were used for adult food and bolts of *A. pensylvanicum* (15-20 cm × 4.7 cm diameter pieces of trunks from saplings) were used for oviposition. Detailed rearing protocols are described in Ugine et al. (2011). Only adults were used in our studies. Prior to all experiments beetles were maintained at 16:8 h light:dark, with 20°C days and 18°C nights. During experiments beetles were maintained at 14.5:9.5 h light:dark and all behavior experiments were conducted during daylight hours. Prior to experiments, beetles were maintained individually in 473 ml 12-cm diameter lidded clear plastic deli cups containing striped maple twigs. During experiments, beetles were maintained as above or were placed in temperature gradient cages. Beetles averaged  $13.6 \pm 0.2$  (mean  $\pm$  SE) d old when starting the no-choice temperature exposure treatment,  $11.1 \pm 0.2$  d old when starting the behavioral fever response experiment, and  $22.7 \pm 0.8$  d old when starting the

thermoregulation after chilling experiment.

## 2.2 Fungal inoculum and general bioassay procedures

The F-52 isolate of *M. brunneum* (ARSEF 7711; USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY), originally isolated from *Cydia pomonella* collected in Austria (Dubois, 2008), was utilized for all fungal inoculations. This strain is the active ingredient in the commercial pesticide Tick-Ex G (EPA Registration Number 72098-12; Novozymes Biologicals, Salem, VA). All beetles were inoculated using fungal bands produced by impregnating non-woven fiber band material (polyester quilt batting) with *M. brunneum* cultures using the procedure described in Shanley et al. (2009). Fungal bands were stored for up to 1 yr at 4°C before use. Conidial concentration per unit area of band material was quantified as in Peng et al. (2011) by blending 5 × 5 cm sections of fungal bands in 0.2% Tween-80, filtering the suspension through two layers of cheesecloth, rinsing the cheesecloth with water to remove conidia, vortexing the filtrate, and counting conidia with a hemocytometer. To quantify conidial viability, the suspension was diluted with water to 1 × 10<sup>7</sup> conidia/ml and a 1 ml aliquot was spread onto each of three 90-mm diameter Petri dishes containing Sabouraud dextrose agar plus yeast extract (Inglis et al., 2012) with 0.1% gentamicin added before autoclaving to control growth of contaminants. The dishes were incubated at 20°C for 12-16 h in the dark and germination, evidenced by the presence of a germ tube, was recorded for 100 conidia at each of three locations on each plate (total = 300 conidia). The average viable conidial concentration for bands used in the behavioral fever experiment was  $7.66 \times 10^6 \pm 1.12 \times 10^6$  viable conidia/cm<sup>2</sup>. Bands used in the no-choice

experiment had an average of  $1.14 \times 10^7 \pm 1.54 \times 10^6$  viable conidia/cm<sup>2</sup>. Both concentrations are above the LC50 of  $3.08 \times 10^6$  viable conidia/cm<sup>2</sup> (Shanley et al., 2009).

Beetles were inoculated by holding them firmly on top of a 5 × 5 cm piece of fungal band for 30 seconds. During inoculation, beetles were held so that the ventral side of the abdomen was in contact with the band surface. Control beetles were pressed onto clean band material. After treatment, beetles were maintained in lidded plastic cups containing striped maple twigs (see above) and were checked daily for mortality for 30 d. After death, cadavers were placed into individual cups at 100% RH and checked for *M. brunneum* outgrowth and sporulation.

### 2.3 Effect of temperature on *M. brunneum* growth

The radial growth of *M. brunneum* under seven temperature regimes was determined by modifying the procedure outlined in Ouedraogo et al. (1997). Six-mm disks of unsporulated mycelium from 2-3 d old cultures were placed in the centers of 90-mm diameter Sabouraud dextrose agar plates. The plates were incubated in darkness at constant temperatures of 23, 28, 31 or 34°C, or at 28, 31 or 34°C for 5 h/d and 23°C for the remainder of the day. Five-hour exposures were used because the temperature rarely exceeds 34°C for longer than 5 h/d in northeastern North America (NOAA, Northeast Regional Climate Center, 1996-2010). Each replicate consisted of five Petri dishes of each treatment and the experiment was conducted four times. Surface radial growth was recorded on day 12 by measuring fungal growth in two directions on the bottom of the plate.

### 2.4 *Anoplophora glabripennis* no-choice temperature exposures

Beetles were inoculated with *M. brunneum* as described above and cups containing individual beetles and striped maple twigs were placed into incubators and held at 23, 28, 31 or 34°C for 5 h and 23°C for 19 hours. The same incubators were used for each temperature for all three reps to prevent variability in temperature exposure between reps. It took the incubators that were programmed to 31°C and 34°C for 5 h longer to warm up than those set at 23 and 28°C. To ensure that all beetles were exposed for 5 h to the respective temperature, the time it took the incubators to reach the warmer temperature was not included in the 5h exposure period. The experiment was repeated three times with 10 male and 10 female inoculated beetles and 3 male and 3 female control beetles for each replicate (total inoculated = 240, total controls = 72).

## 2.5 Testing for behavioral fever and thermoregulation

Temperature gradients were created by placing aluminum foil on the tops of 30.5 × 30.5 × 61.0 cm aluminum screen cages (Fig. 1), with a 20-cm diameter 100 watt heat lamp on top of the foil at one end of the cage and a 30.5 × 14 cm frozen ice pack on top of the foil at the other end. Light leakage from the heat lamp was prevented by wrapping a 76.2 × 15.2 cm strip of aluminum foil around the back top rim of the cage where the lamp was located. Four 50-cm long, 5-15 mm diameter striped maple twigs were hung horizontally 1 cm beneath the top of the cage as a food source and perch by inserting them through wire loops at the ends of the cage. The ambient room temperature was 23°C. The heat lamp area in contact with the cage and ice packs were adjusted as needed to maintain a 20-45°C gradient for 8 h. Beetles had ample ability to maintain temperatures at ambient room temperature (23°C) as the temperature gradient was

localized on cage tops while the rest of the cage was at ambient or close to ambient temperature.

Single beetles were placed in each cage and separate cages were reserved for males and females. Between trials the high contact areas (the inside of the cage top and the upper 5 cm of all sides) were wiped with 70% ethanol. Between experimental replicates, interiors of cages were scrubbed with 6.5% sodium hypochlorite solution and rinsed with water. Each beetle was tested on days 2, 5 and 8 after fungal inoculation and assigned a specific set of twigs. These twigs were placed in the gradient cage when it was occupied by the beetle and then removed and stored in plastic bags at 4-5°C when that beetle was not in the cage.

Beetles were assigned to one of three treatments: 1) inoculated with *M. brunneum* and exposed to a temperature gradient, 2) not inoculated and exposed to a temperature gradient and 3) inoculated and held at constant 23°C. The temperature gradient experiment was conducted with four fungal-inoculated and four untreated females and four fungal-inoculated and four untreated males, for 4 experimental replicates, resulting in a total of 32 fungal-treated and untreated males and females (total = 64 beetles). For each replicate, 8-10 females and 8-10 males were inoculated and held at 23°C (38 males and 38 females).

After inoculation, all beetles (fungus-treated and controls) were held in individual cups at 23°C and 100% RH for 24 h. Beetles inoculated with *M. brunneum* and kept at 23°C were always in cups while beetles exposed to temperature gradients were in cages on days 2, 5, and 8. To reduce differences between these environments, whenever beetles were in gradient cages, wet cotton balls were added to the constant 23°C cups and solid

lids were replaced with mesh lids, and cages and cups were misted twice on each of those days. On days when the gradient beetles were not in cages, they were held in 473 ml cups with twigs and solid lids, the same as the 23°C beetles.

Before temperature gradients were established, beetles for cage treatments were allowed to acclimate within cages at 23°C for 15-24 h. After acclimation, beetles were exposed to gradients for 8 h. Thoracic temperature recordings began once gradients were established. Temperature recordings were taken by measuring the air temperature 1 mm from the side of the thorax using a hypodermic temperature probe (HYPO-331TG60SMPWM; Omega Engineering, Inc., Stamford, Connecticut). Temperature readings were taken every 15 min from 6:30-8:30 am (9 readings) and from 1:30-2:00 pm (3 readings) on days 2, 5 and 8 after fungal infection. Temperature data could only be collected when the beetles were stationary.

To determine whether external temperature readings were indicative of the internal beetle temperature, 8 males and 7 females were held at 23-24°C and 6 males and 4 females were held at 30-34°C. After 15 min their external temperatures were recorded as described above, after which the internal body temperature was taken by inserting the hypodermic temperature probe into the thorax. The temperature 1 mm from thoraxes of acclimated beetles did not differ significantly from the internal thoracic temperatures of beetles held at either temperature (23-24°C: paired t-test,  $t = -1.1456$ ;  $df = 14$ ;  $P = 0.1356$ ; 30-34°C:  $t = -0.36112$ ;  $df = 9$ ;  $P = 0.36315$ ).

## 2.6 Thermoregulation by healthy *A. glabripennis* after chilling

*Anoplophora glabripennis* adults were either maintained at constant 23°C or at

15°C only during the 9.5 h scotophase and 23°C during photophase, before being placed into temperature gradient cages. To test whether food availability impacts temperature choice, adults exposed to either temperature regime were provided with food or no food when inside temperature gradient cages. For trials with food, twigs suspended beneath cage tops were provided as described above. For trials without food, twigs were substituted by 0.95-cm diameter wooden dowels, which were not eaten by these beetles. A single beetle was released into the center bottom of each cage (19-21°C) as soon as photophase began and allowed to acclimate for 30 min before temperature recordings started, as preliminary trials indicated that little movement occurred during the first 30 min after introduction into cages. Beetle temperature was then recorded every 20 min for a total of 13 readings (= 260 min). Between trials, cages and wooden dowels were cleaned with 70% ethanol to remove frass and any contact pheromones, and twigs were replaced. The experiment was repeated 10 times, for a total of 10 males and 10 females per treatment.

## 2.7 Data analysis

The final radii of fungal colonies on day 12 were analyzed using a linear regression model and Tukey's HSD test, with log radius as the response variable and replicate and temperature treatment as explanatory variables (JMP 9.0.02, SAS Institute, 2010). Survival times for beetles in the no-choice temperature experiment and the behavioral fever and thermoregulation experiment were compared using proportional hazards models with censoring at 30 d. Time to death was quantified by recording the day each beetle was found dead and subtracting 0.5 because mortality was checked once a

day. Control beetles were excluded from both analyses as only 4.2% of controls in the no-choice temperature experiment died (and fungal outgrowth did not develop from control cadavers) and no controls in the thermoregulation experiment died. Survival curves and median days to death for both experiments were calculated using Kaplan-Meier survival analysis. A proportional hazards regression (PHREG) was used for generating all pair-wise comparisons among treatments in the no-choice temperature experiment (SAS Institute, 2008; 2010).

For beetles in the behavioral fever and thermoregulation experiment, mean morning and afternoon thoracic temperatures were calculated for each beetle on each experimental day. A multilevel model was used, with experimental day and time of day as repeated measures for individual beetles (SAS Institute, 2010). The dependent variable was average temperature and explanatory variables were replicate, individual beetle, sex, treatment, time of day and experimental day. Experimental replicate and individual beetles were coded as random and individual beetles were nested within replicate. Beetle temperatures in the thermoregulation after chilling experiment were analyzed using a multilevel model because beetles were measured repeatedly. Experimental replicate and individual beetle were coded as random, beetles were nested within replicate and time was coded as a continuous variable. The effects of sex, time, temperature exposure (15 vs. 23°C) and food availability and the interactions between sex, time and food availability were included in the model.

### 3. Results

#### 3.1 Effect of temperature on *M. brunneum* growth



Temperature had a significant effect on final average radius of *M. brunneum* cultures ( $F = 1597.7030$ ;  $df = 6, 6$ ;  $P < 0.0001$ ) (Fig. 2). When exposed to constant 28°C, *M. brunneum* exhibited the greatest final average radius compared to all other temperatures (Tukey's HSD;  $P \leq 0.05$ ). However, average radius declined rapidly when fungi were constantly exposed to temperatures warmer than 28°C (Tukey's HSD;  $P \leq 0.05$ ). Overall, 5 h exposures to 31°C and 34°C were not as detrimental to fungal growth as constant exposures to 31 or 34°C (Tukey's HSD;  $P \leq 0.05$ ). *M. brunneum* exposed to 34°C for 5 h/d grew an average of  $13.6 \pm 0.2$  mm in radius while *M. brunneum* cultures constantly exposed to 34°C grew an average of only  $0.5 \pm 0.2$  mm in radius.

### 3.2 *Anoplophora glabripennis* no-choice 5 h temperature exposures

The interaction between temperature and sex was not significant and was removed from the model. *Anoplophora glabripennis* infected with *M. brunneum* and maintained at 34°C for 5 h/d lived significantly longer than fungal-infected beetles at 31, 28 or 23°C for 5 h/d ( $\chi^2 = 98.001$ ;  $df = 1$ ;  $P < 0.0001$ ). Fungal-infected beetles held at 34°C for 5 h/d lived a median of 14.5 d (95% CI: 13.5, 17.5) while those at 31, 28 or 23°C for 5 h/d lived a median of 12.5 d (CI: 11.5, 12.5) (Fig. 3). Males lived significantly longer than females ( $\chi^2 = 7.4629$ ;  $df = 1$ ;  $P = 0.0063$ ) (males: median 13.5 d, CI: 12.5, 14.5; females: median 12.5 d, CI: 11.5, 13.5). Fungal outgrowth was evident on 93.3% of the cadavers of fungal-treated beetles.

### 3.3 Testing for behavioral fever and thermoregulation

The interaction between sex and treatment was not significant and was removed

from the model. Fungal-infected *A. glabripennis* caged in temperature gradients did not increase their body temperatures above temperatures chosen by healthy controls in gradients ( $F = 0.8816$ ;  $df = 1, 57.89$ ,  $P = 0.3517$ ). There was no significant difference between the temperatures chosen by males or females ( $F = 3.2486$ ;  $df = 1, 57.52$ ;  $P = 0.0767$ ). The mean temperatures chosen by infected and healthy temperature gradient beetles did not differ in the morning versus afternoon ( $F = 1.0470$ ;  $df = 1, 289.40$ ;  $P = 0.3070$ ) or by experiment day ( $F = 0.2914$ ;  $df = 2, 307.30$ ;  $P = 0.7474$ ).

The interaction between sex and treatment was not significant when the dependent variable was days to death and was removed from the model. Fungal-infected beetles placed in temperature gradients on days 2, 5, and 8 post-inoculation lived significantly longer (medium 13.5 d, CI: 10.5, 14.5) than infected beetles held at constant 23°C (median 11.3 d, CI: 10.5, 12.5) ( $\chi^2 = 5.1006$ ;  $df = 1$ ;  $P = 0.0239$ ). There was no significant effect of sex but there was a trend towards females living longer than males ( $\chi^2 = 3.6148$ ;  $df = 1$ ;  $P = 0.0573$ ) (males: median 11.5 d, CI: 10.5, 12.5; females median 12.5 d, CI: 10.5, 13.5). After death, fungal outgrowth was evident on 94.5% of cadavers of fungal-treated beetles and none of the control beetles died.

### 3.4 Thermoregulation by healthy *A. glabripennis* after chilling

Prior chilling 15°C compared to beetles held constantly at 23°C had no effect on temperatures subsequently chosen by beetles placed in temperature gradient cages, whether provided with food ( $F = 1.8428$ ;  $df = 1, 29.31$ ;  $P = 0.1850$ ) or not ( $F = 2.3919$ ;  $df = 1, 26.36$ ;  $P = 0.1339$ ), so prior temperature exposure was removed from models. The model terms sex, time in the gradient (0.5 to 4.8 h) and food interacted significantly in

this test of thermoregulation after chilling, so subsequent analyses were conducted separately on beetles provided with food or not (SAS Institute, 2010). Time and sex interacted significantly for beetles provided with food ( $F = 25.6153$ ;  $df = 1, 344.9$ ;  $P < 0.0001$ ). Males with food significantly increased their average temperature over the 4.3 h observation period ( $F = 48.5227$ ;  $df = 1, 176.9$ ;  $P < 0.0001$ ,  $3.0 \pm 0.7^{\circ}\text{C}$  increase) while females with food did not significantly change their average temperature ( $F = 0.0068$ ;  $df = 1, 167.8$ ;  $P = 0.9344$ ) (Fig. 4A). For beetles without food, time was significant ( $F = 17.2699$ ;  $df = 1, 356.4$ ;  $P < 0.0001$ ) but sex was not ( $F = 0.0047$ ;  $df = 1, 26.44$ ;  $P = 0.9459$ ). Without food both sexes increased their temperature over time an average of  $2.4 \pm 0.8^{\circ}\text{C}$  (Fig. 4B).

Figure 1. Temperature gradient cage.

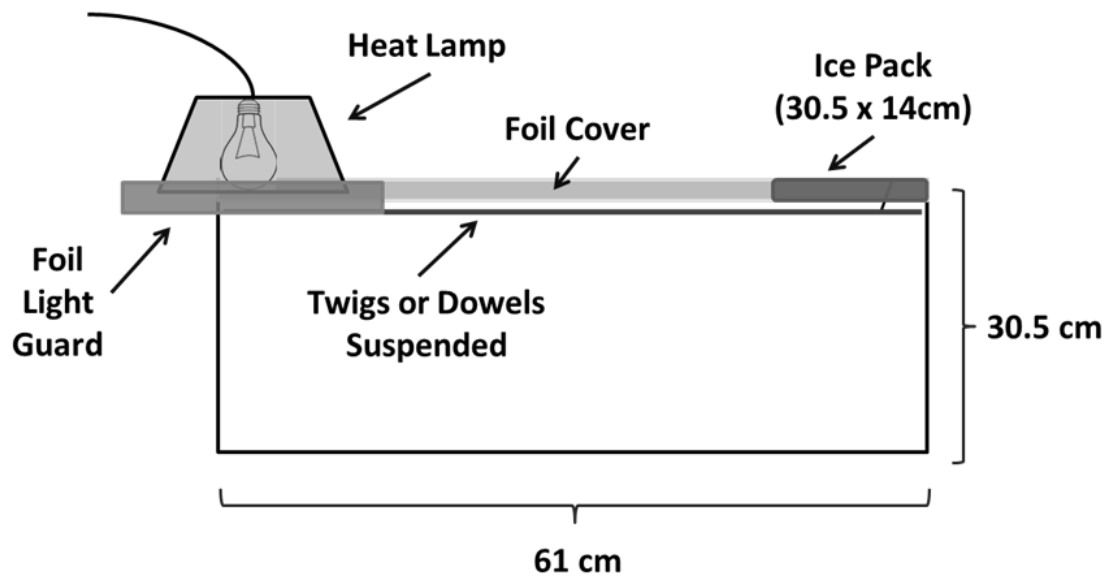


Figure 2. Effect of temperature on the average radius (mm  $\pm$  SE) of *M. brunneum* after 12 d. Fungal cultures were incubated in constant darkness at either constant 23, 28, 31 or 34°C or 5 h/d at 28, 31 or 34°C and 23°C for the remainder of the day. Bars with different letters are significantly different (Tukey's HSD;  $P \leq 0.05$ ).

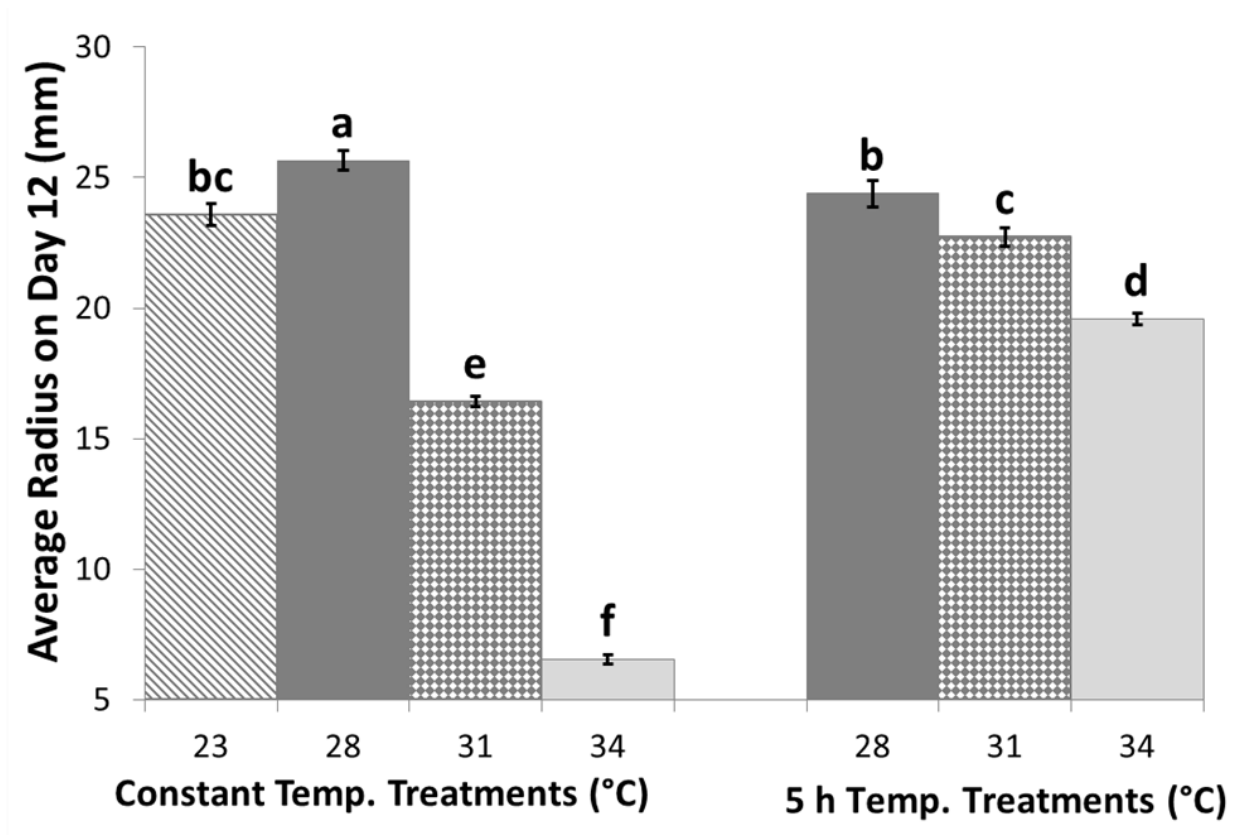


Figure 3. Percentages of beetles surviving over time for males and females (merged) treated with *M. brunneum* and held for 5 h/d at 23, 28, 31 or 34°C and 23°C for the rest of the day. Control beetles held at each temperature were combined because there was no significant difference in the time to death ( $\chi^2 = 4.6299$ ;  $df = 3$ ;  $P = 0.2010$ ).

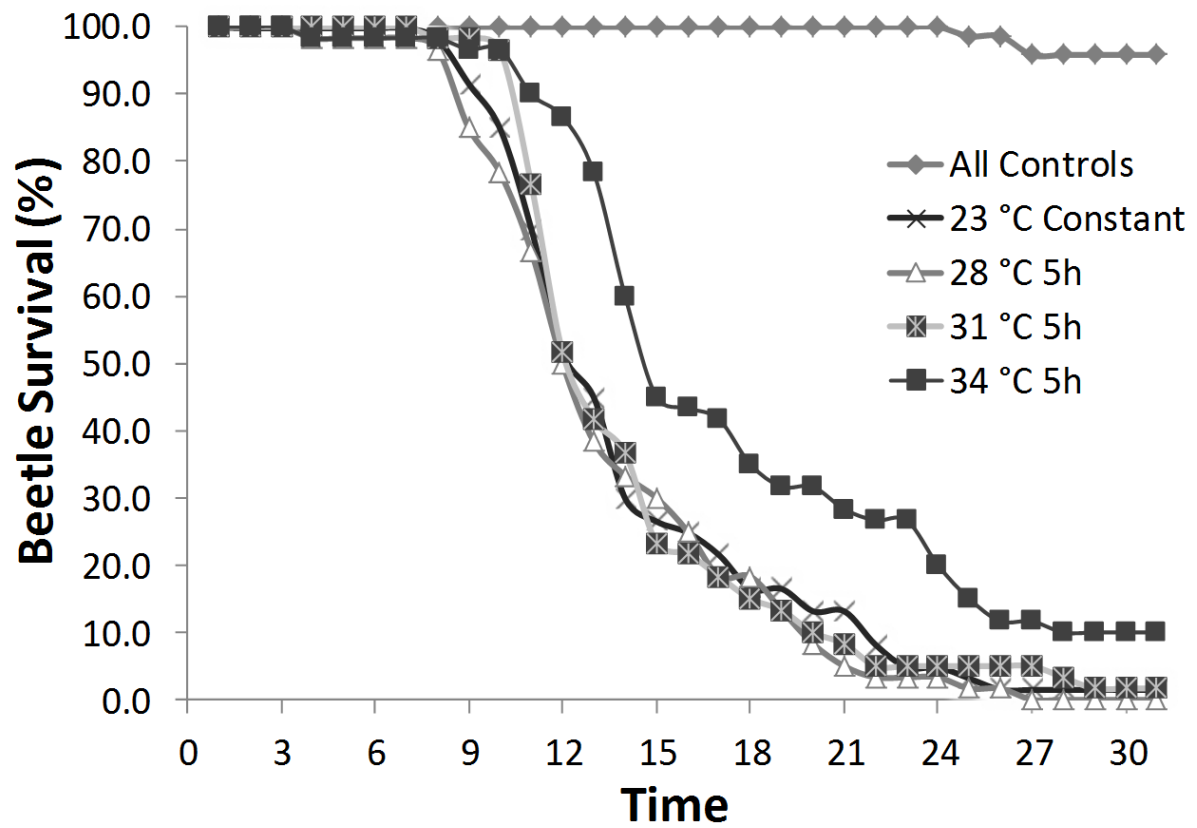
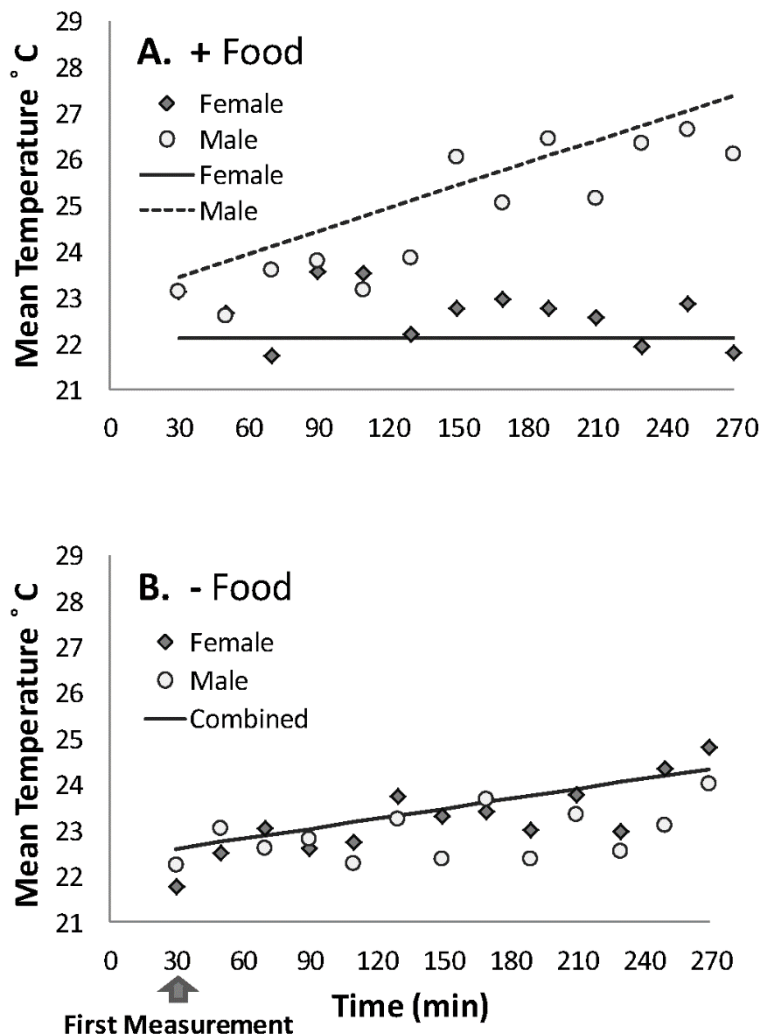


Figure 4. A. Mean temperatures of healthy males and females over time provided with food (+ Food). There was a significant effect of time for males ( $F = 48.5227$ ;  $df = 1$ , 176.9;  $P < 0.0001$ ) but no significant effect of time for females ( $F = 0.0068$ ;  $df = 1$ , 167.8;  $P = 0.9344$ ). B. Merged mean temperature of healthy males and females over time not provided with food (- Food). Among these beetles there was a significant effect of time ( $F = 17.2699$ ;  $df = 1$ , 356.4;  $P < 0.0001$ ) but not sex.



## 4. Discussion

Adult *A. glabripennis* infected with *M. brunneum* did not exhibit behavioral fever, although doing so could have significantly lengthened their survival time. Holding *M. brunneum*-infected *A. glabripennis* at 34°C for 5 h/d significantly increased their survival. We hypothesize this was because fungal growth was negatively affected by higher temperatures. In vitro, cultures of *M. brunneum* grew fastest when held at constant 28°C, while in vitro growth of this isolate was inhibited at higher temperatures, in agreement with Foster et al. (2010). We found not only temperature but also length of exposure to high temperatures affects *M. brunneum* growth in vitro. Constant 31 and 34°C significantly reduced fungal growth compared to 5 h exposures to these temperatures but even 5 h exposures to these higher temperatures slowed growth compared with 28°C. We hypothesize that because in vitro fungal growth was inhibited by higher temperatures fungal growth in vivo was also inhibited at 34°C. It is also possible that higher temperatures could delay or inhibit sporulation. Among six isolates of *M. anisopliae* only 26-67% of the conidia germinated within 24 h at 34 °C while at lower temperatures (20-30 °C) over 80% of the conidia germinated within 24 h (Dimbi et al., 2004). We hypothesize that a delay in conidial germination or reduction in conidial viability would probably result in fewer conidia penetrating the insect cuticle thereby reducing the dose received by the insect.

Higher temperatures are also known to increase insect immunity by enhancing phagocytosis (Ouedraogo et al., 2003) and the activity of phenoloxidase (Adamo and Lovett 2011; Catalán et al., 2012) and antimicrobial activity in the hemolymph. In some cases this increase in immunity can lead to increased resistance to some pathogens



including fungi. Therefore it is also possible that infected beetles held at 34°C lived longer because fungal growth was not simply inhibited but the beetle's protective immune response was increased at higher temperatures.

Although *A. glabripennis* did not exhibit behavioral fever when infected, males provided with food and starved males and females significantly increased their preferred body temperature over time, independent of prior cold exposure. Females are synovigenic (i.e., continue to produce and mature eggs throughout their lives) and it could be that female beetles increased their preferred body temperature over time when denied access to food because they were actively searching for food. In lower density populations, these polyphagous beetles are found in tree canopies where they have ready access to ample food, unless suitable trees are scarce or overcrowded, at which time they would disperse to find food (Hu et al., 2009). So, we hypothesize that it is unlikely that females in lower density populations would often need to increase temperature preference over time. It is not known why males have a different response than females.

In our experiment, chilled *A. glabripennis* did not exhibit basking behavior (i.e., position themselves to increase their body temperatures above the normothermic levels of healthy individuals) after being placed in the temperature gradients. This suggests *A. glabripennis* are not strict thermoregulators and, as such, may have a limited capacity to utilize temperature as a defensive mechanism (Thomas and Blanford, 2003). It may be possible that behaviors leading to presence of *A. glabripennis* adults at tops of trees in the morning, as reported in China (T. Poland, personal communication), could principally be light-seeking rather than heat-seeking. Results from this study did not address light-seeking behavior as the design of our studies was only based on detecting responses to

temperatures and eliminated possible phototropism.

The failure of *A. glabripennis* to exhibit a fever response to *M. brunneum* infection could either suggest fevering may be a specific rather than a general response to some pathogens, or that *A. glabripennis* may not be adapted to fever at all. In some cases, insects exhibit a fever response to certain pathogens but not to others. The cricket *A. domesticus* exhibited behavioral fever when inoculated with *R. grylli* but not when inoculated with the bacterium *Serratia marcescens* (Bizio), parasitoid flies or gregarine gut parasites (Adamo, 1998). Among these parasites and pathogens, only *R. grylli* was negatively affected by warmer temperatures. Lack of fever adaptation can also be a result of pathogen and host mismatch. Meadow grasshoppers (*Chorthippus parallelus* Zetterstedt) failed to exhibit behavioral fever when exposed to *B. bassiana* or *M. acridum* (Springate and Thomas, 2005). However, normal thermoregulatory behavior by *C. parallelus* that constrained the development of the native pathogen *B. bassiana* had no effect on the non-native pathogen *M. acridum*. Additionally, it is possible that the fungal pathogen itself may be inhibiting behavioral fevering. *M. brunneum* produces the toxin destruxin A, which has been shown to interfere and significantly shorten the behavioral fever response in *S. gregaria* (Hunt and Charnley, 2011; Wang et al., 2012).

Negative fitness costs associated with high temperatures could put selective pressure on *A. glabripennis* to avoid high temperatures, preventing them from exhibiting a fever response even when it would appear to be beneficial. Fevering has been shown to be metabolically costly to the host: *M. domestica* infected with a high dose of *B. bassiana* fevered longer and at a higher temperature than house flies infected with a low dose, suggesting that these insects perhaps tailor their behavioral fevering response to delay

mortality while also reducing the cost of fevering (Anderson et al., 2013b). Additionally, fevering may not result in an increase in fitness or may affect other aspects of insect physiology. For example, fevering reduced *S. gregaria* males' flight capacity and ability to compete for mates (Elliot et al., 2005). *Beauveria bassiana*-infected *M. domestica* that fevered laid almost twice as many eggs as insects that did not fever, but had no net increase in fecundity because egg viability was reduced (Anderson et al., 2013a).

Infected male *A. glabripennis* lived significantly longer by 1 d than infected females in the no-choice experiment but no significant difference was seen in the behavioral fever study. We hypothesize that the difference in these results is most likely due to experimental variation. In previous studies, there was no effect of sex on mean time to death for *M. brunneum* F-52-infected *A. glabripennis* (Dubois et al., 2008. Shanley et al., 2009).

In our study, we did not observe a significant effect of temperature on uninfected control beetle survival, but we did not measure fecundity and experiments were terminated after 30 d (beetles can live for several months), so we may not have detected longer-term fitness costs due to exposure to higher temperatures. It would be interesting in the future to examine whether or not short term (5 h) high temperature exposures would decrease beetle fecundity, especially as Keena (2006) reported that that with constant exposures to  $> 24^{\circ}\text{C}$  *A. glabripennis* longevity and fecundity declined and at  $\geq 35^{\circ}\text{C}$  oviposition was arrested. Regardless, we found that adult *A. glabripennis* infected with *M. brunneum* will be unlikely to escape fungal biological control through thermoregulation as infected beetles did not exhibit behavioral fever, although doing so could have significantly lengthened their survival time.

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## Chapter 3. Maternal exposure of a beetle to pathogens protects offspring against fungal disease

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## Abstract

Maternal exposure to an immune challenge can convey enhanced immunity to invertebrate offspring in the next generation. We investigated whether maternal exposure of the Asian longhorned beetle, *Anoplophora glabripennis*, to two species of the fungus *Metarhizium* or the bacterium *Serratia marcescens* elicited transgenerational immune priming (TGIP). We tested specificity of this protection and whether occurrence of TGIP was dependent on maternal exposure to living versus dead pathogens. Our results show that TGIP occurred and protected offspring against *Metarhizium brunneum*. Maternal exposure to *S. marcescens* provided non-specific protection to offspring against a fungal pathogen, but TGIP in response to *Metarhizium* only occurred when offspring were exposed to the same fungal species that was used to prime mothers. Moreover, TGIP in response to *M. brunneum* occurred only after maternal exposure to living rather than dead fungus. Our findings suggest that occurrence of TGIP could be both specific and dependent on whether the pathogen was alive

Key words: transgenerational immune priming, insect immunity, maternal effects, fungi, bacteria, entomopathogen

# 1. Introduction

In invertebrates, exposure to an immune challenge (a pathogen or pathogen-derived factor) can enhance the immune response to a subsequent challenge, a phenomenon known as immune priming (Kurtz and Armitage, 2006). Enhanced immunocompetence can persist throughout development within individuals and can even be conveyed to offspring in the next generation, an effect called trans-generational immune priming (TGIP) (Huang and Song, 1999; López et al., 2001; Moret and Schmid-Hempel, 2001; Reber and Chapuisat, 2012; Thomas and Rudolf, 2010). Parental exposure to bacteria and bacterial cell wall components, (Freitak et al., 2009, 2014; Little et al., 2003; López et al., 2001; Moret, 2006; Moret and Schmid-Hempel, 2001; Roth et al., 2010; Sadd et al., 2005; Zanchi et al., 2011, 2012), a DNA virus (Huang and Song, 1999; Tidbury et al., 2011), microsporidia (Lorenz et al., 2011) or fungal cell walls (Huang and Song, 1999) can enhance offspring immunity and in some cases even offspring survival.

The degree to which a parental pathogen challenge provides specific protection (i.e., offspring are protected against only the same pathogen experienced by the parent) versus non-specific protection (i.e., offspring are protected against a pathogen not experienced by the parent) varies in different systems. Fungal pathogens used to challenge mothers provided non-specific protection to offspring against a virus (Huang and Song, 1999) and a protist (Lorenz et al., 2011). In contrast, in a different system offspring were only protected when exposed to the same bacteria experienced by the mother (Roth et al., 2010).

Few studies have taken into account whether exposure to a living pathogen is necessary for TGIP to occur. The majority of studies of TGIP in insects have been based

on heat-killed pathogens injected into insect hosts, thereby bypassing the cuticular or gut defenses which a pathogen would normally have to overcome during the infection process. The use of heat-killed pathogens also prevents exposure of the insect to the cascades of pathogen-produced compounds including proteases and virulence factors characterizing a normal infection (Vega and Lacey, 2012). In *Drosophila* both fungal cell wall components and virulence factors are necessary to fully activate the host's immune response against fungal entomopathogens through the Toll pathway which is involved in recognizing fungi and gram positive bacteria (Chamy et al., 2008; Gottar et al., 2006; Valanne et al., 2011). *Drosophila melanogaster* individuals mutant for the protease involved in detecting fungal virulence factors were more susceptible to fungal pathogens that secrete these virulence factors than flies that were able to detect these virulence factors (Gottar et al., 2006). Several studies have used living pathogens to prime insects within a generation (Freitak et al., 2009; Kurtz and Franz, 2003; Lorenz et al., 2011; Reber and Chapuisat, 2012; Rodrigues et al., 2010; Tidbury et al., 2011). However, only two studies have investigated TGIP in response to living pathogens, including living virus (Tidbury et al., 2011) and living bacteria (Little et al., 2003) but not living fungi, and we are aware of no studies directly comparing whether priming resulted from exposure to living versus dead pathogens.

We investigated TGIP in the Asian longhorned beetle, *Anoplophora glabripennis*, a wood borer native to China and Korea which has been introduced to Europe and northeastern North America (Brabbs et al., 2015; Haack et al., 2006; Hu et al., 2009). This long-lived wood borer takes 1-2 years for larvae to develop and adults often live for more than a month. This beetle is under eradication everywhere that it has been

introduced because it has the potential to cause extensive damage in urban and natural forests if it becomes established. The entomopathogenic fungus *Metarhizium brunneum* is pathogenic to *A. glabripennis* and is being developed as a means for biological control. We were interested in determining if a prior maternal pathogen exposure could make *M. brunneum* less effective in controlling *A. glabripennis* offspring.

We conducted bioassays to investigate 1.) whether maternal exposure to a living compared to a dead fungal pathogen (*M. brunneum*) conveys protection to *A. glabripennis* offspring, and 2.) the extent to which the primed response in offspring is specific to the pathogen experienced by mothers. We found variable specificity in TGIP in response to fungal versus bacterial pathogens. Additionally, only maternal exposure to a living fungal pathogen increased offspring resistance to that pathogen and exposure to heat-killed fungal pathogens did not elicit TGIP.

## 2. Materials and Methods

### 2.1 Insect culture

*Anoplophora glabripennis* were reared and tested in a quarantine facility (APHIS permit P526P-14-00458; Cornell University Sarkaria Arthropod Research Laboratory). Striped maple (*Acer pensylvanicum*) was used for adult food and oviposition. Detailed rearing protocols for adults and larvae are described in Ugine et al. (2011). Only adults were challenged with pathogens in this study. Prior to experiments, beetles were maintained at 14:10 h light:dark, with 25°C days and 16°C nights, individually in 473 ml lidded clear plastic cups containing striped maple twigs. The age of female beetles used for the maternal priming treatments averaged  $7.7 \pm 0.1$  d (mean  $\pm$  SE) post-eclosion at the



beginning of the experiment. Offspring weight and whether or not offspring successfully completed development and eclosed as F1 generation adults were recorded. Mothers were exposed to either living *M. brunneum* or *M. anisopliae*, or to dead *M. brunneum* or *S. marcescens* and all offspring were challenged with *M. brunneum*.

## 2.2 Fungal preparation

The F-52 isolate of *M. brunneum* (ARSEF 7711; USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY) and the closely related *M. anisopliae* isolate ESC1 (ATCC 32176; American Type Culture Collection, Manassas, VA) were grown at 26°C on potato dextrose agar (PDA) (Difco, Sparks, MD) with 0.1% gentamicin to control growth of contaminants.

Living fungal suspensions were prepared by washing conidia off of cultures using 0.05% Tween 80 and 2 mm glass balls to dislodge and suspend conidia. Conidial viability was quantified by spreading a 100 µl aliquot of  $1 \times 10^7$  conidia/ml onto each of 360-mm diam petri dishes containing PDA and incubating plates overnight at 26°C, after which they were examined to determine whether or not conidia were viable, indicated by the presence of germ tubes.

For treatment with a dead pathogen, conidia of *M. brunneum* were harvested from PDA plates and placed in an oven at 100°C for 1h, to kill the conidia, which were then harvested as above. The success of the heat-killed treatment was confirmed by plating the conidial suspension on PDA and incubating overnight at 26°C. The vaccine was prepared by suspending the heat-killed conidia in Grace's Insect Media (GIM) (BioWhittaker, Walkersville, MD), and the suspension was then stored at -80°C until use.

## 2.3 Bacterial preparation

*Serratia marcescens* was grown in Luria Bertani (LB) broth (Klein, 1997) overnight at ambient temperatures on a rotating shaker at 175 rpm. A portion of the inoculated broth was serially diluted, plated on Luria Bertani Agar (LA) and incubated overnight at 26°C to estimate colony forming units (CFUs)/ml. Bacteria in the remainder of the broth were heat-killed at 95-100°C for 20 min. The success of the heat-kill treatment was confirmed by plating the bacterial suspension on LA and incubating overnight at 26°C.

## 2.4 Maternal inoculations with *M. brunneum* or *M. anisopliae*

Beetles that would become mothers were exposed to either living *M. brunneum* or *M. anisopliae* or dead *M. brunneum*. For exposures to living fungal pathogens, fungal suspensions used to inoculate beetles were adjusted to  $10^3$  conidia/ml using 0.05% Tween 80. The doses of living fungi were limited by the virulence of the fungal pathogen because the experiment depended on the female beetles living long enough to reproduce. Females were inoculated by gently shaking them in 15 ml of a suspension of living conidia of either *M. brunneum* or *M. anisopliae* for 5 sec (solutions were vortexed for 15 sec prior to beetle inoculation). The average concentrations of viable conidia for *M. brunneum* and *M. anisopliae* suspensions were  $9.56 \times 10^2 \pm 1.44 \times 10$  conidia/ml and  $9.63 \times 10^2 \pm 5.61 \times 10$  conidia/ml, respectively, after accounting for conidial viability. This experiment was repeated 3 times, with *M. brunneum* (n=5) and *M. anisopliae* (n=5) inoculated female beetles in each replicate.

For treatment with the dead fungal pathogen, female beetles were injected with heat-killed *M. brunneum*. Female beetles were cold-anesthetized for 8 min, rinsed in 70% ethanol and injected with 10 µl of the heat-killed conidial suspension in the intersegmental membrane between the metasternum and the rear coxal cavity using a 3 ml syringe mounted in a syringe microburet (Micro-Metric Instrument Co, Cleveland, OH). The conidial suspension was vortexed for 15 sec before loading into the syringe. Control beetles were injected with 10 µl of sterile GIM. The average injected dose across replicates was  $4.24 \times 10^6 \pm 3.40 \times 10^5$  conidia/ml (Rep 1:  $4.03 \times 10^6 \pm 1.28 \times 10^6$ ; Rep 2:  $3.78 \times 10^6 \pm 1.64 \times 10^5$ ; Rep 3:  $4.90 \times 10^6 \pm 4.63 \times 10^5$  conidia/ml). The experiment was repeated 3 times with female *M. brunneum*-injected beetles (n=5) and female GIM control beetles (n=5) for each replicate.

## 2.5 Maternal inoculations with *S. marcescens*

Female beetles were injected with 10 µl of  $10^7$  CFU/ml heat-killed *S. marcescens* in sterile LB broth. Control beetles were injected with 10 µl of sterile LB broth. The experiment was repeated 3 times with *S. marcescens*-injected beetles (n=5) and female broth control-injected beetles (n=2) for each replicate.

## 2.6 Rearing offspring

All treated females were mated with an untreated male beetle 6 d after pathogen inoculation. The one male that died during the experiment was incubated after death to confirm that it did not become infected with fungi acquired when mating with a fungal-treated female; the fungus did not sporulate from the cadaver, so it is unlikely that the

male died of fungal infection. All pairs of beetles were maintained separately in 3.8 l glass jars containing 7 striped maple twigs and a bolt of striped maple and allowed to oviposit for 2 weeks. After each week, bolts were replaced. After 2 weeks males and females were removed from jars and maintained separately in 473 ml lidded clear plastic cups containing striped maple twigs and checked daily for mortality for 60 d.

## 2.7 Offspring bioassays with living *M. brunneum*

Offspring hatching from eggs laid by treated females were reared to adult as described before. Adults from this F1 generation were challenged with living *M. brunneum* when used in offspring bioassays. The beetles averaged  $22.3 \pm 0.2$  d post-eclosion at the start of the bioassays. Conidial viabilities were determined by applying a 10  $\mu$ l aliquot of  $10^7$  conidia/ml cottonseed oil to a PDA plate and placing a coverslip on the drop to ensure that the conidia in oil contacted the agar. Using the resulting percent germination to quantify conidial viability, conidial suspensions were adjusted to  $10^7$  viable conidia/ml using cottonseed oil.

Offspring from both the maternal treatment and control groups were inoculated by placing a 2  $\mu$ l drop of  $10^7$  conidia/ml on the inter-segmental membrane between the thorax and abdomen. Following inoculation the beetles were checked daily for death over 72 days. Bioassays were conducted 3 times with offspring from each maternal pathogen treatment (*S. marcescens* n = 62; heat-killed *M. brunneum* n = 87; living *M. brunneum* n = 51; living *M. anisopliae* n = 51; Table S1 in File S1) and with offspring from each maternal control treatment (naive n = 60; broth injection n = 27; GIM injection n = 52). The 60 offspring from the naive maternal control treatment were used as untreated

negative controls and received a drop of sterile cottonseed oil instead of being inoculated with *M. brunneum*.

## 2.8 Data analysis

Bioassays ended at 72 days and living offspring were censored for analysis. Survival times for offspring were compared using proportional hazards models (SAS Institute Inc., 2010). All non-significant interactions were removed from the models. The explanatory variables were replicate, sex, and maternal treatment. Survival curves and median days to death were calculated using Kaplan-Meier survival analyses.

Among offspring in bioassays, the times to death for beetles in each treatment were compared to the respective controls. If the treatment effect was significant then treatment levels were analyzed using risk ratios with alpha values adjusted using the Bonferroni correction to take into account multiple comparisons.

Failure of larvae and pupae to complete development and eclose was analyzed using a linear regression model. The proportion of offspring for each mother that failed to eclose was normalized using a log (x +1) transformation. The explanatory variables were replicate, maternal treatment, and female weight.

## 3. Results

We found evidence of TGIP for offspring challenged with *M. brunneum* whose mothers were treated with the gram negative bacterium *S. marcescens*, as these offspring lived significantly longer than controls ( $\chi^2_2 = 23.57$ ,  $p < 0.0001$ ; risk ratios,  $p \leq 0.0167$ , Table 1, Fig. 5). Maternal exposure to *S. marcescens* increased offspring survival by a

median of 15-16.5 d compared to controls (median days to death: *S. marcescens*, 42 d (95% CI: 36, 46); naive control, 25.5 d (CI: 23, 34); broth control, 27 d (CI: 19, 37)). There was no effect of sex for time to death of offspring whose mothers were either exposed to *S. marcescens* or received a control treatment ( $\chi^2_1 = 1.99$ ,  $p = 0.1585$ ).

Additionally, we found evidence of immune priming for offspring challenged with *M. brunneum* whose mothers were treated with living *M. brunneum* ( $\chi^2_1 = 9.07$ ,  $p = 0.0026$ , Fig. 6). These beetles lived significantly longer than naive controls (median days to death: living *M. brunneum*, 29 d (CI: 27, 37); naive control, 25.5 d (CI: 23, 34)). There was no effect of sex for offspring whose mothers were either exposed to living *M. brunneum* or received the control treatment ( $\chi^2_1 = 0.22$ ,  $p = 0.6363$ ). Offspring challenged with *M. brunneum* whose mothers were injected with heat-killed *M. brunneum* or inoculated with living *M. anisopliae* did not live significantly longer than the respective controls (heat-killed *M. brunneum*,  $\chi^2_2 = 0.74$ ,  $p = 0.6896$ , Fig. S1; living *M. anisopliae*,  $\chi^2_1 = 0.13$ ,  $p = 0.7216$ , Fig. S2). There was no effect of sex for offspring whose mothers were exposed to heat-killed *M. brunneum* or offspring exposed to living *M. anisopliae* or their respective controls (heat-killed *M. brunneum*,  $\chi^2_1 = 0.38$ ,  $p = 0.5375$ ; living *M. anisopliae*,  $\chi^2_1 = 0.36$ ,  $p = 0.5465$ ). Interactions between sex and treatment for all models were not significant and were removed. There was no effect of maternal treatment or weight on whether or not offspring failed to complete development and eclose (treatment  $F_{5, 69} = 0.40$ ,  $p = 0.85$ ; female weight  $F_{1, 69} = 1.19$ ,  $p = 0.28$ ).

Figure 5. Survival curves for offspring of the *S. marcescens* treatment. Percentages of male and female (merged) offspring treated with *M. brunneum* surviving over time whose mothers were challenged with either *S. marcescens* or a control treatment (naive control or broth control). Different letters signify significant differences in survival curves between treatments (risk ratios,  $p \leq 0.0167$ ).

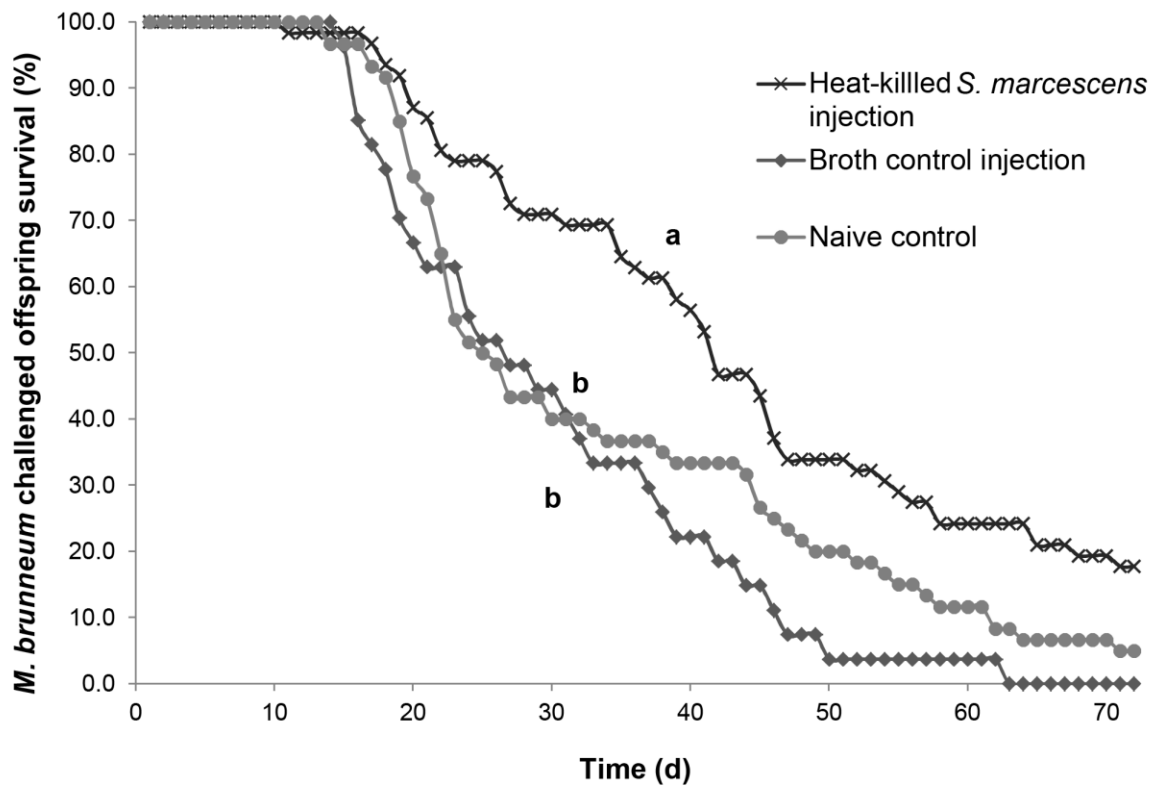


Figure 6. Survival curves for offspring of the live *M. brunneum* treatment. Percentages of male and female (merged) offspring treated with *M. brunneum* surviving over time whose mothers were challenged with either a living dose of *M. brunneum* or a control treatment (naive control). Different letters signify significant differences in survival curves between treatments ( $\chi^2_1 = 9.07$ ,  $p = 0.0026$ ).

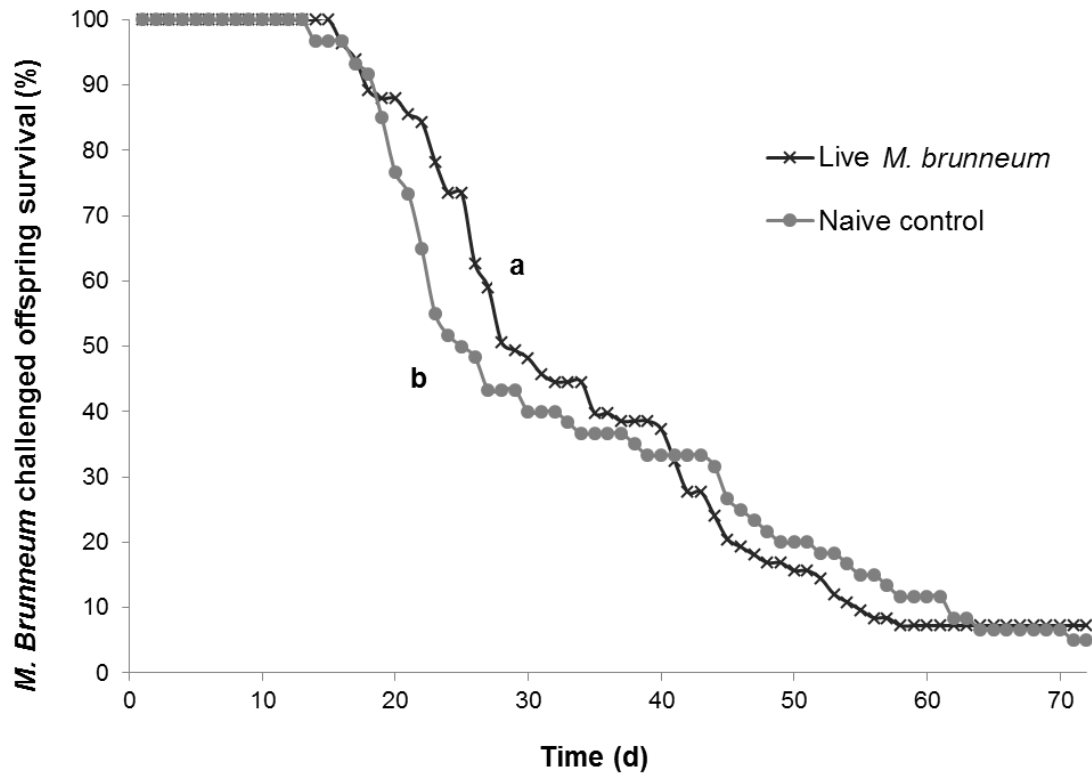




Table 1. Offspring (F1 generation) bioassay experimental design and results. Statistics were generated using proportional hazards models and risk ratios adjusted using a Bonferroni correction for multiple comparisons ( $\alpha=0.0167$ ).

Primed	Offspring Challenge	Controls	Model	$\chi^2$	df	p-value	TGIP <sup>1</sup> ?	Pair-wise comparisons	risk ratio	p-value $\alpha=0.0167$
Heat-killed <i>S. marcescens</i>	Living <i>M. brunneum</i>	Naive, Broth Injection	Treatment	23.57	2	< 0.0001	Yes	<i>S. marcescens</i> vs Naive	0.4726	0.0004
			Sex	1.99	1	0.1585		<i>S. marcescens</i> vs Broth Injection	0.2919	< 0.0001
			Rep	107.7	2	< 0.0001		Naive vs Broth Injection	0.6176	0.0478
Heat-killed <i>M. brunneum</i>	Living <i>M. brunneum</i>	Naive, GIM <sup>2</sup> Injection	Treatment	0.74	2	0.6896	No			
			Sex	0.38	1	0.5375				
			Rep	144.8	2	< 0.0001				
Living <i>M. brunneum</i>	Living <i>M. brunneum</i>	Naive	Treatment	9.07	1	0.0026	Yes			
			Sex	0.22	1	0.6363				
			Rep	101.4	2	< 0.0001				
Living <i>M. anisopliae</i>	Living <i>M. brunneum</i>	Naive	Treatment	0.13	1	0.7216	No			
			Sex	0.36	1	0.5465				
			Rep	88.17	2	< 0.0001				

<sup>1</sup>TGIP = Transgenerational Immune Priming, <sup>2</sup>Grace's Insect Media.

## 4. Discussion

We found that TGIP was dependent on whether mothers were exposed to a living or dead fungal pathogen, i.e., offspring of mothers exposed to living but not heat-killed *M. brunneum* lived longer than controls when challenged with *M. brunneum*. Only two other studies have investigated TGIP with a fungal pathogen. Priming in mosquitoes was found in response to living fungi (Lorenz et al., 2011), as is consistent with our findings. However, Huang and Song (Huang and Song, 1999) found TGIP in shrimp in response to glucans, which are components of all fungal cell walls. Glucans would have been present in the cell walls of heat-killed *M. brunneum* but we did not find priming in response to heat-killed *M. brunneum*. Although these former studies found TGIP in response to living or dead pathogens, we are aware of no other studies that have made a direct comparison between a living versus dead challenge with the same pathogen species.

We hypothesize that heat-killed *M. brunneum* did not induce TGIP because it lacked the compounds produced during an active fungal infection. There is some evidence that substances produced during pathogen growth are necessary for priming to occur, at least within a generation. For example, Milutinović et al. (2014) found that for larvae of the flour beetle *Tribolium castaneum*, challenged with the gram positive bacterium *Bacillus thuringiensis*, priming within a single generation only occurred when larvae were exposed to spore supernatants containing substances produced during bacterial growth. Priming however did not occur when larvae were exposed to autoclaved or heat-killed pathogen cells that would have lacked these substances (Milutinović et al., 2014).

In our study, it is also possible that differences in the methods used for inoculation with living versus dead pathogens could have influenced results. The heat-killed pathogens were injected into the insects' hemocoel, while the living fungal pathogens were applied externally to the beetles' cuticle. The heat-killed pathogen was injected to ensure the pathogen would elicit the insects' immune response as these insects exhibit very limited grooming behavior (JJF unpublished data) so they would not become inoculated with topical application. In contrast, the living fungal pathogen was applied externally because if this pathogen was injected, insects would have been killed before they could reproduce. The insect cuticle provides not only mechanical protection but is also an active biochemical barrier with antibacterial compounds and the ability to inhibit the growth of fungal germ tubes before they enter the hemocoel (Moret and Moreau, 2012). Therefore it is possible that differences in time to death among treatments with living versus dead pathogens could be influenced in part by the fact that heat-killed fungi and bacteria were injected into the insect and bypassed this defensive barrier while living fungal pathogens had to enter through the cuticle.

Maternal exposure to fungi only provided specific protection when both mothers and offspring were exposed to the same isolate of *M. brunneum*, but not when mothers were exposed to *M. anisopliae* and offspring were challenged with *M. brunneum*. A similar specific TGIP response was also seen in *T. castaneum* exposed to bacteria (Roth et al., 2010). Offspring of either paternally or maternally primed *T. castaneum* adults had enhanced survival when exposed to the same bacterial challenge as their parent but not when exposed to a different bacterium. Inducing a specific transgenerational immune response would be advantageous if the next generation will encounter the same pathogen

as the parental generation and specificity would be advantageous in this circumstance as immune priming can be costly for both mothers and offspring (Contreras-Garduño et al., 2014; Roth et al., 2010; Sadd and Schmid-Hempel, 2009; Trauer and Hilker, 2013 Zanchi et al., 2011). Maternal immune priming can result in reductions in maternal and offspring fecundity, slower development time in offspring or decreased offspring developmental time resulting in a decrease in pupal weight (Contreras-Garduño et al., 2014; Roth et al., 2010; Sadd and Schmid-Hempel, 2009; Trauer and Hilker, 2013 Zanchi et al., 2011). The potential costs that can be associated with priming make it especially important that TGIP provides protection to offspring against pathogens they are likely to encounter. However, in this study we saw no effect of maternal treatment on the ability of offspring to eclose, regardless of treatment, although it is possible that there are other costs present in this system which we did not investigate. Our findings agree with those of Freitak et al. (2009) and Zanchi et al. (2011) who also found no decrease in offspring survival to adulthood after maternal exposure to a bacterial challenge.

Unlike maternal exposure to fungal pathogens, maternal exposure to heat-killed *S. marcescens* provided non-specific protection to offspring challenged with *M. brunneum*. However it is not known whether exposure to heat-killed *S. marcescens* would provide specific protection to offspring because offspring were not challenged with *S. marcescens*. TGIP conveying non-specific protection to offspring has been observed in other systems as well (Huang and Song, 1999; Lorenz et al., 2011). Thus, we found variable specificity in TGIP against two entomopathogens in the fungal genus *Metarhizium* and maternal exposure to the gram negative bacterium *S. marcescens* protected offspring against a fungal pathogen. In *Drosophila* fungi and gram negative

bacteria induce different pathways in the insect immune response. Gram negative bacteria primarily induce the IMD pathway while fungi primarily induce the Toll pathway (Lemaitre and Hoffman, 2007). However in *T. castaneum* both a gram positive and a gram negative bacterial species as well as fungus induced both the Toll and IMD pathways (Yokoi et al., 2012). This demonstrated that the induction of these pathways by microbes is more promiscuous in *T. castaneum* than in *Drosophila*. It may be that heat-killed *S. marcescens* led to TGIP because of the cross-talk between the Toll and IMD pathways in coleopteran insects. It could be that heat-killed *S. marcescens* provided greater protection to offspring than *M. brunneum* because of differences in how these pathogens induce the insect's immune response.

## 5. Conclusions

We found evidence that maternal exposure to a living compared to a dead fungal pathogen was necessary to convey protection to offspring of *A. glabripennis*. We speculate this was because the heat-killed *M. brunneum* lacked the compounds normally produced during fungal infection that would induce priming. We also found that offspring were only protected with maternal exposure to the same fungal pathogen that was experienced by the offspring and maternal exposure to *S. marcescens* bacteria provided non-specific protection to offspring against a fungal pathogen.

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Chapter 4. Influence of mating and age on susceptibility of the  
beetle *Anoplophora glabripennis* to the fungal pathogen  
*Metarhizium brunneum*

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## Abstract

The age and life history of an insect can influence its susceptibility to pathogens. Reproduction can be costly and may trade off with immunity while it is generally assumed that immunity will decrease with increasing age through a process called immunosenescence. Fungal pathogens are used as biological control agents for a variety of insect pests, and *Metarhizium brunneum* is being developed to control the Asian longhorned beetle (*Anoplophora glabripennis*), an invasive wood-borer. Because adult female *A. glabripennis* take 1-2 weeks to mature after eclosion and both sexes can be long-lived, we investigated how age and mating status would influence susceptibility of *A. glabripennis* to *M. brunneum*. Young (6.5 day-old) unmated, mature (27-33 day-old) mated and unmated, and old (57-71 day-old) unmated and mated adults were inoculated with a lethal dose of *M. brunneum*. The presence of *M. brunneum* in the hemolymph was quantified and beetle mortality was monitored daily. There was a cost to reproduction for mated mature male and female beetles which died a median of 1.6-1.9 days earlier than unmated beetles, while there was no effect of mating on susceptibility for old beetles. We found no evidence for immunosenescence in old beetles, as they did not die faster than young or mature beetles. Young unmated males however were more susceptible than mature or old unmated males, while there was no effect of age on susceptibility of unmated females.

Key words: *Anoplophora glabripennis*, *Metarhizium brunneum*, invertebrate immunity, aging, reproduction, immunosenescence

## 1. Introduction

Reproduction is energetically costly and since immune defense can also be costly (Sheldon & Verhulst, 1996) there may be tradeoffs between immunity and reproduction (Lawniczk et al, 2006). These tradeoffs can lead to increased susceptibility to a pathogen challenge if invertebrates maximize their overall fitness at the expense of being able to mount an effective immune response (Fedorka et al., 2004; Krams et al., 2013; Schmid-Hempel, 2003; Siva-Jothy et al., 2005). Mating reduced several aspects of the immune response in the striped ground cricket, *Allonemobius socius* (Fedorka et al., 2004), and the Japanese calopterygid damselfly, *Matrona basilaris japonica*, had reduced ability to encapsulate foreign objects following copulation and oviposition (Siva-Jothy et al., 1998). The transfer of sperm and seminal proteins, including “sex peptide”, during mating increased the susceptibility of female *Drosophila melanogaster* to *Pseudomonas aeruginosa*, *Providencia rettgeri* and *Providencia alcalifaciens* (Fedorka et al., 2007; Short and Lazzaro, 2010), while male *D. melanogaster* challenged with *Escherichia coli* showed reduced immunocompetence when mated with multiple females compared to a single female (McKean and Nunney, 2001). Mated *Tenebrio molitor* had reduced phenoloxidase (PO) activity compared with unmated beetles (Rolff and Siva-Jothy, 2002).

Immunological tradeoffs with reproduction and other fitness traits have also been observed in selection experiments in which selection for enhanced reproduction and fitness frequently leads to a reduction in immune competence and vice versa. Selection for earlier reproduction or high intensity sexual selection, such as when the sex ratio is

male-biased (polyandry), increased the cost of reproduction leading to a decrease in immunocompetence in some species (reviewed in Siva-Jothy et al., 2005; Van Lieshout et al., 2014). Selection of insects for resistance to bacteria, a granulovirus, the malaria parasite, nematodes and parasitoids have all led to reduced fitness, including increased larval mortality, slower development, and reduced reproductive success and fecundity (reviewed in Siva-Jothy et al., 2005). The evidence from these studies further supports experimental findings that reproduction and immunity are costly and are frequently traded off.

The age of an invertebrate can also influence its susceptibility to a pathogen challenge (DeVeale et al., 2004; Stanley, 2012). As animals age the immune system can break down and have a diminished capacity to properly react and combat a pathogen challenge (immunosenescence) (Stanley, 2012). A decline in immunity with increasing age has been found in female and male *Tribolium castaneum* beetles (Khan et al., 2016), cabbage white butterflies, *Pieris rapae* (Stoeckl, 2007), male *T. molitor* beetles (Daukste et al., 2012) and workers of two species of bumblebees, *Bombus terrestris* and *Bombus lucorum* (Doums et al., 2002). This area, however, needs further investigation as older leaf-cutting ants, *Acromyrmex octospinosus* (Armitage & Boomsma, 2010), honey bees, *Apis mellifera* (Bull et al., 2012), and *Drosophila melanogaster* (Khan and Prasad, 2013), did not show a decline in immunity with increasing age.

The fungal pathogen *M. brunneum* is being developed to control the Asian longhorned beetle, *Anoplophora glabripennis* and has been found to not only kill beetles but also reduce the fecundity of females by decreasing both the number and viability of eggs which are laid (Goble et al., 2015; Hajek and Bauer 2009; Hajek et al., 2008; Hu et

al., 2009). This insect is an invasive wood-boring cerambycid native to China and Korea that was initially detected in North America in 1996 (Haack et al., 2010; Hu et al., 2009). It has a very wide host range consisting of many species of hardwood trees and is an economically important pest in the US, Canada and Europe, where it is currently under eradication (Brabbs et al., 2015; Haack et al., 2010; Hu et al., 2009). These beetles are long-lived, as adult males live for 99-106 d and adult females live for 73-88 d (Keena, 2002; Smith et al., 2002), while larvae take 1-2 years to develop within trees (Haack et al., 2006). After eclosion beetles are not reproductively mature until they are 9-15 days old after which both sexes will mate repeatedly (Keena, 2002; Keena and Sánchez, 2006; Smith et al., 2002). Mature females lay eggs throughout their lives (Keena, 2002; Hajek et al. 2008).

We investigated how age and mating influenced susceptibility of *A. glabripennis* to *M. brunneum*. We inoculated Young (6.5 days old) unmated, Mature (27-33 days old) mated and unmated, and Old (57-71 days old) mated and unmated adult beetles with lethal doses of *M. brunneum*. The age range for the oldest age group was chosen to challenge female beetles that would be a few weeks from dying if healthy. Mortality of challenged beetles was monitored daily and the presence of *M. brunneum* cells in the hemolymph on days 5 and 9 after inoculation was quantified. We hypothesized that mating would increase the susceptibility of beetles to *M. brunneum* and that older beetles would be more susceptible to *M. brunneum* than younger beetles.

## 2. Materials and methods

### 2.1 Insect culture



*Anoplophora glabripennis* were reared and tested under quarantine and USDA APHIS permit at Cornell University's Sarkaria Arthropod Research Laboratory. Striped maple (*Acer pensylvanicum*) was used for adult food and oviposition. Detailed rearing protocols for adults and larvae are described in Goble et al. (2015) and only adults were used in this study. Prior to the experiment, beetles were maintained at 16:8 h light:dark, with 23°C days and 16°C nights, individually in 540 ml, 7.9 x 9.3 cm h/w lidded clear plastic cups containing striped maple twigs changed weekly. Mated pairs were maintained in 2,312 ml, 11.5 × 16 cm h/w clear plastic containers containing striped maple twigs before treatment.

#### 2.1.1 Beetle age groups and mating regimes

Beetles in three age groups were exposed to *M. brunneum* to determine whether susceptibility changes with age (Fig. 7). Beetles in the Young age group averaged  $6.5 \pm 0.0$  (mean  $\pm$  SE) d after eclosion. Mature beetles averaged  $30.6 \pm 0.1$  d after eclosion and old beetles averaged  $63.4 \pm 0.4$  days after eclosion. The effect of mating and age on beetle susceptibility to *M. brunneum* was tested by exposing Mature and Old unmated and mated beetles to *M. brunneum*. Beetles in the Young age group were too young to mate because at this age they will fight rather than mate (unpublished results). For this reason only unmated Young beetles were included and therefore they could only be compared to unmated Mature and Old beetles.

To mate beetles, Mature beetles were paired with the opposite sex at  $17.5 \pm 0.4$  d after eclosion and Old beetles were paired at  $19.9 \pm 0.3$  d after eclosion. After being paired, Mature beetles remained together for  $12.9 \pm 0.2$  d and Old beetles remained

together for  $43.9 \pm 0.4$  d, after which the pairs of both groups were separated and used in the bioassay or hemolymph assay.

## 2.2 Fungal culture and bioassays

*Anoplophora glabripennis* were exposed to the F-52 isolate of *Metarhizium brunneum* (ARSEF 7711; USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY). Fungal cultures were grown at 26°C on potato dextrose agar (PDA) (Difco, Sparks, MD) with 0.1% gentamicin to prevent growth of contaminants.

Suspensions of inoculum were prepared by washing conidia off of cultures using 0.05% Tween 80 and 2 mm glass balls to dislodge and suspend conidia. Conidial viability was quantified by spreading a 100  $\mu$ l aliquot of  $1 \times 10^7$  conidia/ml onto each of three 60-mm diam petri dishes containing PDA. Plates were incubated for 16-17 h at 26°C, and examined to determine whether conidia were viable, indicated by the growth of germ tubes. Percent conidial viability was evaluated prior to preparation of the conidial suspensions for use in bioassays which were adjusted to  $10^8$  viable conidia/ml using 0.05% Tween.

Pairs of beetles were separated prior to treatment to prevent the transfer of conidia between inoculated insects since lethal doses of conidia can be transferred between mates (Peng et al. 2011; Ugine et al. 2014). Beetles were inoculated by shaking them in 11-13 ml of the *M. brunneum* conidial suspension for 5 sec (solutions were vortexed for 15 sec prior to beetle inoculation). Control beetles were shaken in 13 ml of sterile 0.05% Tween. After treatment, beetles were individually placed in plastic cups as described above and

mortality was monitored daily for 28 d (Fig. 7). Cadavers were checked for fungal outgrowth after death. The experiment was repeated three times with a total of 43-46 beetles in each age group treated with *M. brunneum* and 11-20 beetles in each age group as controls.

### 2.3 Hemolymph assays

Beetles used for the hemolymph assays were inoculated as described above and were bled on days 5 and 9 post inoculation (Fig. 7). Whether or not blastospores (fungal cells) were present in the hemolymph of treated beetles was determined by examining hemolymph samples using a hemocytometer. To collect hemolymph samples, beetles were cold-anesthetized at 4°C for 8 min, rinsed in 70% ethanol, blotted dry with a sterile towel, bled by puncturing the intersegmental membrane between the thorax and abdomen with a sterile insect pin and hemolymph was collected into a 20 µl microcapillary pipette (Kimble Chase, Vineland, NJ). Hemolymph (20 µl) was gently mixed with an equal amount of a 2X concentrated ice-cold protease inhibitor cocktail (cOmplete ULTRA tablets, Roche Diagnostics, Indianapolis, IN) suspended in PBS buffer, pH 6.4. One hemolymph sample per beetle was collected and enough hemolymph was obtained for two hemocytometer counts. The experiment was repeated five times with a total of 36-37 beetles in each age group treated with *M. brunneum* and 22-25 beetles in each control group treated with 0.05% Tween.

### 2.4 Data analysis

All fungal-inoculated beetles died prior to censoring and control beetles alive at

the end of the bioassays were censored at 28 d. Since the Young beetles were too young to mate two separate analyses were conducted. *Anoplophora glabripennis* are difficult and expensive to rear and we did not have enough beetles to use different sets of beetles for different experiments. The mating and age analysis included data for Mature and Old, mated and unmated beetles while the age analysis included data for Young, Mature and Old unmated beetles. Survival times for beetles in the mating and age analysis were normally distributed and were analyzed using a least squares linear regression model. Pair-wise comparisons between treatments were analyzed using Tukey's HSD post-hoc tests (SAS Institute Inc., 2013a). Survival times for beetles in the age analysis were normalized using a  $\ln(x + 0.5)$  transformation and analyzed using PHREG, so that a potential significant interaction between treatment and sex could be investigated (SAS Institute Inc., 2013b). Significant interactions were analyzed using least square means post-hoc tests with alpha levels adjusted using the Bonferroni correction to account for multiple comparisons. The explanatory variables were replicate, sex, and treatment and the median days to death were calculated using Kaplan-Meier survival analyses (SAS Institute Inc., 2013a).

To investigate Old female control mortality, which was greater than Young or Mature control mortality, the survival of control beetles that died versus fungal-inoculated Old female beetles that died was analyzed using a proportional hazards model. The explanatory variables were replicate, treatment (control or fungal-inoculated) and mating status (mated or unmated). All non-significant interactions were removed from the model and the median days to death were calculated using Kaplan-Meier survival analyses.

The presence or absence of detectable levels of blastospores in the hemolymph of fungal-inoculated beetles was analyzed using PROC GENMOD with a binomial distribution and a logit link function (SAS Institute Inc., 2013b). The explanatory variables were replicate, sex, treatment and day after fungal inoculation (day 5 or day 9). Significant interactions were analyzed using least square means post-hoc tests with alpha levels adjusted using the Bonferroni correction to adjust for multiple comparisons.

### 3. Results

#### 3.1. Effect of mating and age

There was a significant effect of age and mating status ( $F_{3,3} = 5.71$ ,  $P = 0.0010$ , Tukey's HSD,  $P \leq 0.05$ ) (Table 2) on how long it took beetles to die. Mature mated beetles were more susceptible to *M. brunneum* and died a median of 1.6-1.9 days faster than Mature and Old unmated beetles, but did not die significantly faster than Old mated beetles [median days to death (Fig. 8): Mature mated, 10.2 d (95% CI: 9.5, 10.2); Mature unmated, 12.1 (10.6, 12.5); Old mated, 11.4 (9.6, 11.4); Old unmated, 11.8 (10.7, 12.6)]. There was a significant effect of sex for Mature and Old beetles ( $F_{1,1} = 17.19$ ,  $P < 0.0001$ ), with females dying one day sooner than males [Female, 10.0 d (9.0, 10.0); Male, 11.0 (10.0, 12.0)] (Fig. 9). The interaction between sex and treatment was not significant and was removed from the model.

Across all bioassays, less than 12% of controls died, excluding the females in the Old treatment groups, and none of the controls that died sporulated. Among female controls there was 44.4% mortality in the Old unmated control treatments and 66.7% mortality in the Old mated treatment group. There was a significant effect of fungal

treatment on Old female beetle mortality as control beetles that died lived significantly longer than Old fungal-exposed females ( $F_{1,1} = 11.80$ ,  $P = 0.0006$ ). Old control beetles lived a median of seven days longer than Old fungal-exposed beetles [median days to death (Fig. S1): Old control, 17 d (95% CI: 10, 21); Old Fungal-exposed, 10 d (95% CI: 9, 10). For beetles that died there was no effect of whether or not beetles had been mated ( $F_{1,1} = 0.54$ ,  $P = 0.4619$ ).

There was a significant interaction between treatment and day for whether or not beetles had blastospores present or absent in sampled hemolymph ( $\chi^2_3 = 12.36$ ,  $P = 0.0062$ ) (Fig. 10; Table 2). Mated Mature and Old beetles were more likely to have blastospores in hemolymph samples on day 9 compared to day 5, but were not more likely to have blastospores in hemolymph samples compared to unmated beetles on either day (Least Squares Means; multiple comparisons,  $P \leq 0.0033$ ). There was a significant effect of sex, and females were more likely to have blastospores present in sampled hemolymph than males (beetles with blastospores in hemolymph (Fig. 11; Table 2): Female,  $45.4 \pm 4.7$  %; Male,  $37.5 \pm 6.4$  %;  $\chi^2_1 = 9.66$ ,  $P = 0.0019$ ).

### 3.2. Effect of age

For the unmated beetles included in this analysis there was a significant interaction between treatment and sex ( $F_{2,124} = 4.65$ ,  $P = 0.0113$ ) (Table 2) regarding how long it took unmated beetles to die after fungal inoculation. Young males were more susceptible and died at least 3 days earlier than Mature or Old male beetles [Least Squares Means (Fig. 12); multiple comparisons,  $P \leq 0.0056$ ; Young males, 8.5 d (8, 10); Mature males, 11.5 d (10, 14); Old males, 13 d (11, 15)]. There was no significant

difference in the susceptibility of females of different ages (Least Squares Means; multiple comparisons,  $P > 0.0056$ ). There was also no significant difference in the susceptibility of Young females compared to Young males (Least Squares Means; multiple comparisons,  $P > 0.0056$ ). Only one Young control died and it did not sporulate. Mortality for the other control groups is discussed above.

There was a significant interaction between treatment and day 5 vs 9 for whether blastospores were detected in the hemolymph of unmated beetles ( $\chi^2_2 = 16.48$ ,  $P = 0.0003$ ) (Fig. 13; Table 2). Young beetles were more likely to have blastospores in their hemolymph on day 9 than on day 5 and to have blastospores present in their hemolymph on day 9 than Old beetles, but not Mature beetles (Least Squares Means; multiple comparisons,  $P \leq 0.0056$ ). There was no effect of sex on presence of blastospores in hemolymph when comparing beetles of the three age groups ( $\chi^2_1 = 1.58$ ,  $P = 0.2085$ ).

Figure 7. Experimental design and sample sizes.

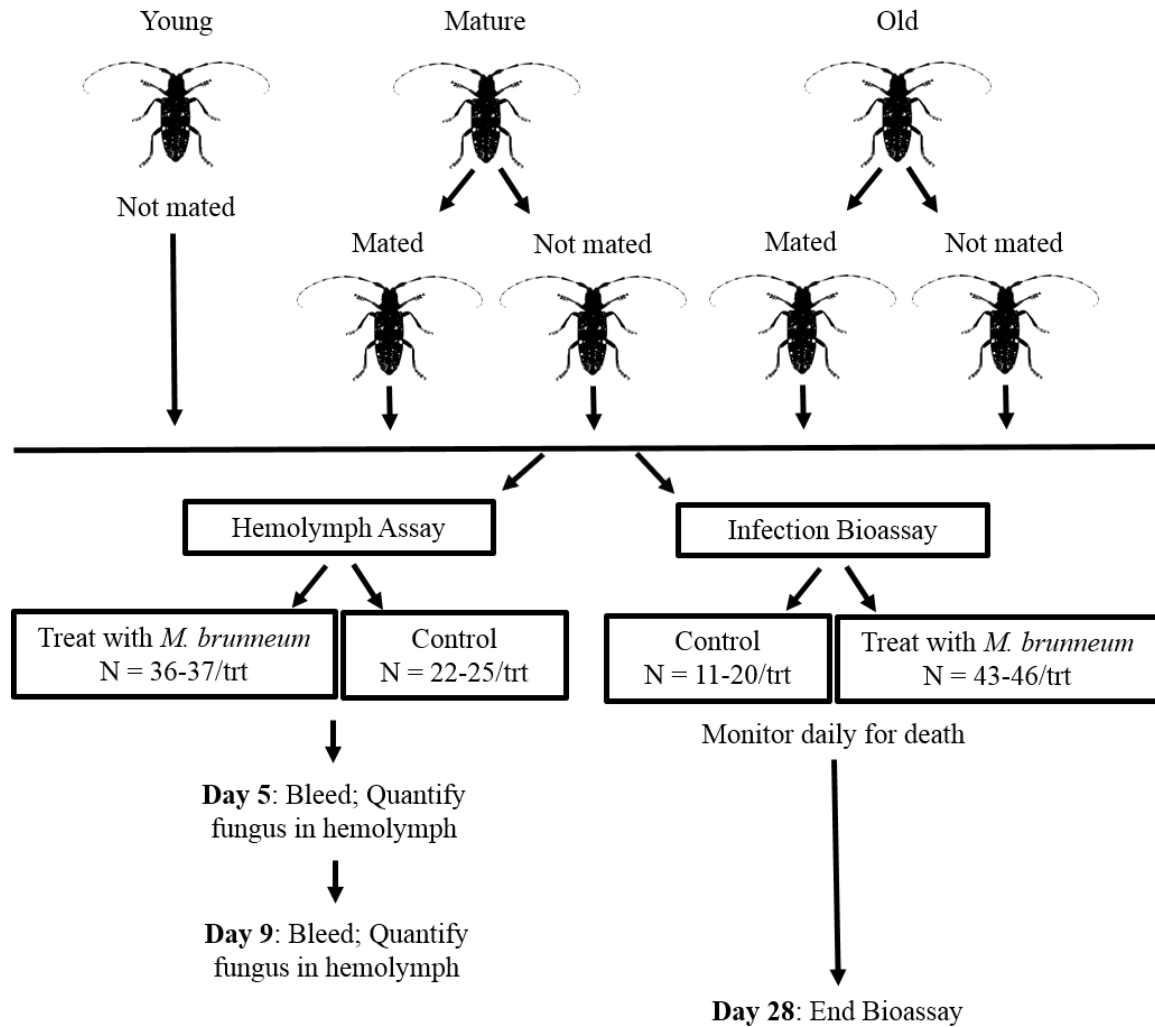




Figure 8. Percentages of beetles (sexes combined) inoculated with *M. brunneum* surviving over time in the mating and age analysis. Different letters indicate significant differences in survival curves between treatments (Tukey's HSD,  $p \leq 0.05$ ).

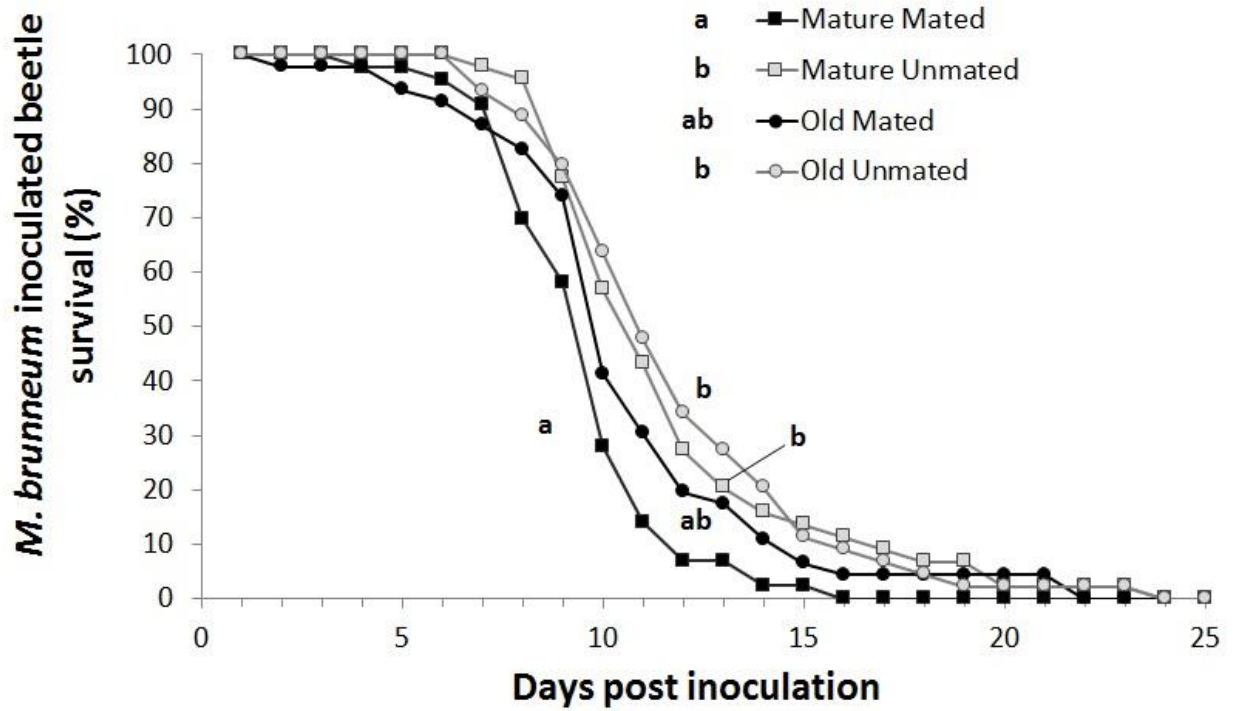


Figure 9. Percentages of male versus female beetles (treatments combined) inoculated with *M. brunneum* surviving over time in the mating and age analysis. Different letters indicate significant differences in survival ( $F_{1,1} = 17.19$ ,  $P < 0.0001$ ).

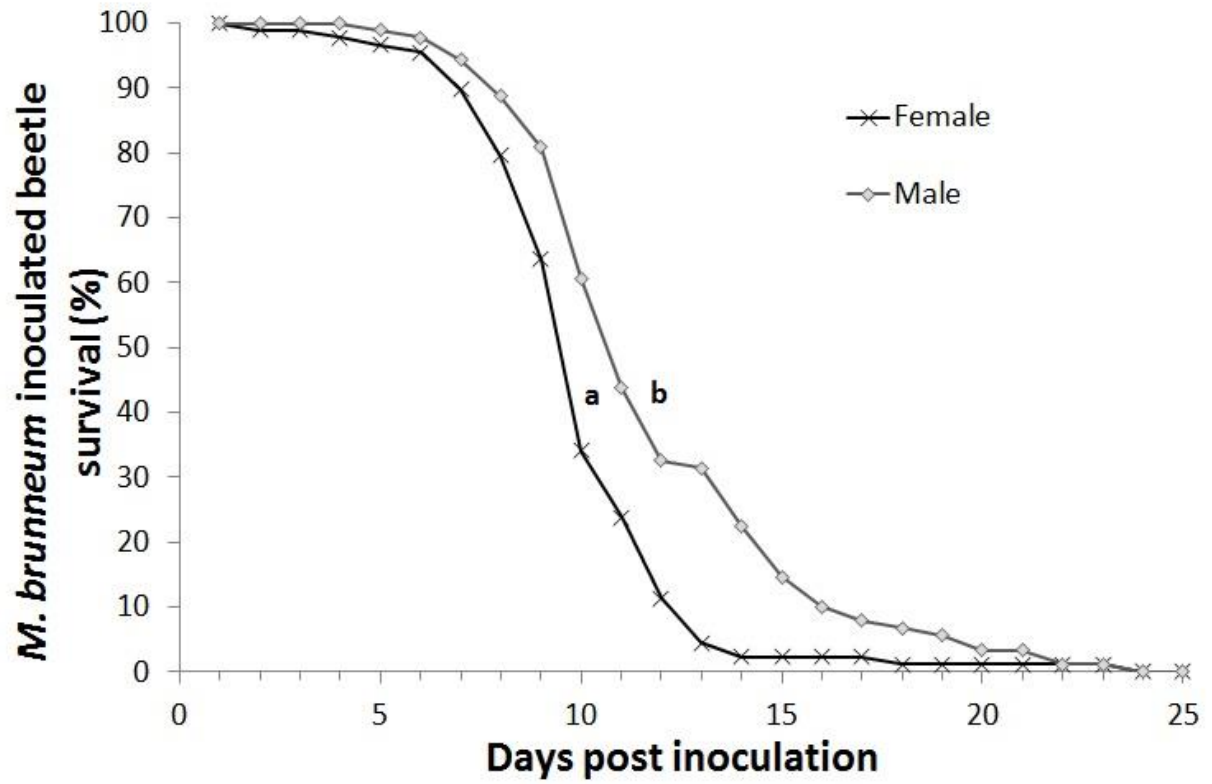


Figure 10. Percentages of beetles (sexes combined) for each treatment in the mating and age analysis inoculated with *M. brunneum* that had blastospores in hemolymph sampled on day 5 versus day 9. Different letters indicate significant differences between treatments (Least Squares Means; multiple comparisons,  $p \leq 0.0033$ ).

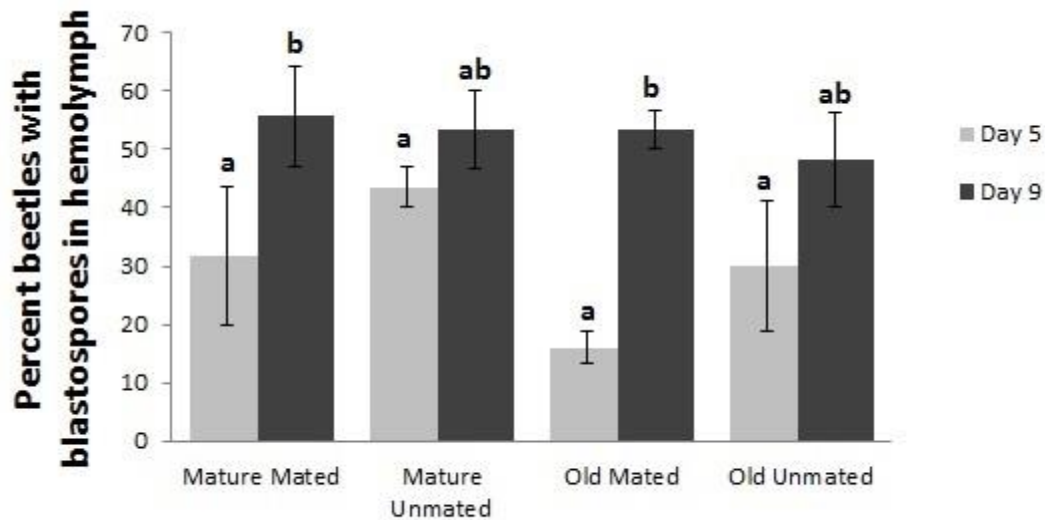


Figure 11. Percentages of male versus female beetles in the mating and age analysis (treatments combined) inoculated with *M. brunneum* that had blastospores in sampled hemolymph. Different letters indicate significant differences ( $\chi^2_1 = 9.66$ ,  $p = 0.0019$ ).

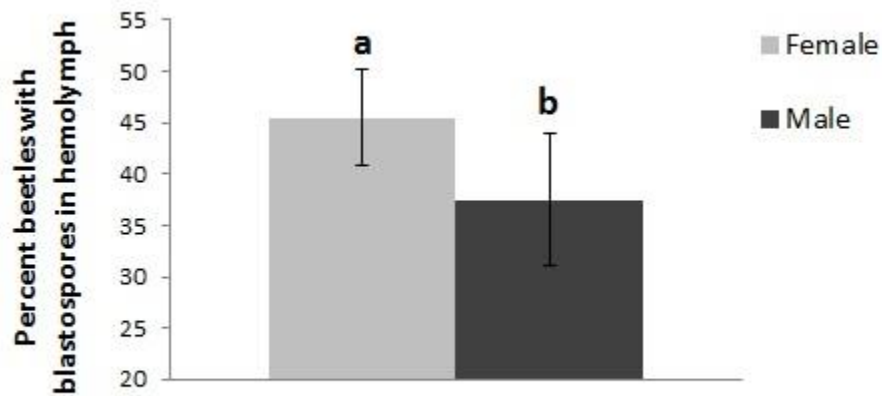


Figure 12. Percentages of male beetles inoculated with *M. brunneum* surviving over time in each treatment of the age analysis. Different letters indicate significant differences in survival curves between treatments (Least Squares Means; multiple comparisons,  $p \leq 0.0056$ ).

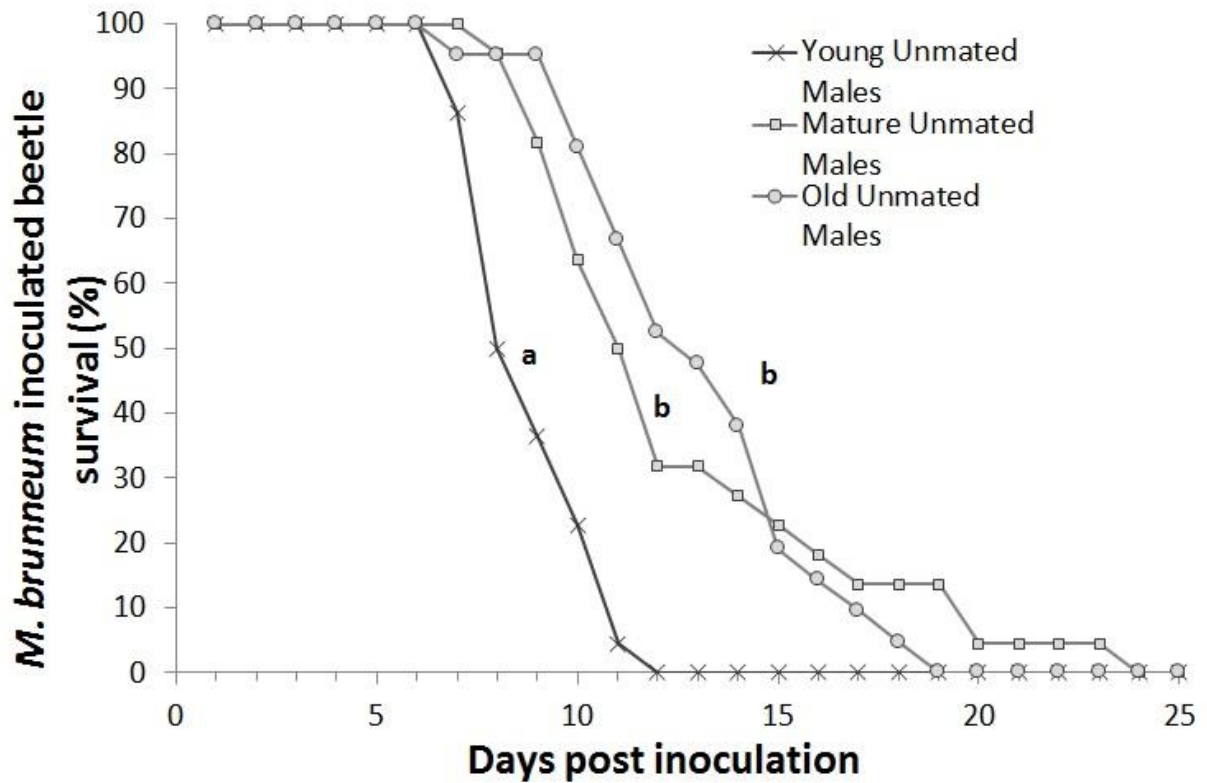


Figure 13. Percent of male and female beetles (combined) for each treatment in the age analysis inoculated with *M. brunneum* which had blastospores in sampled hemolymph on day 5 versus day 9. Different letters indicate significant differences between treatments (Least Squares Means; multiple comparisons,  $p \leq 0.0056$ ).

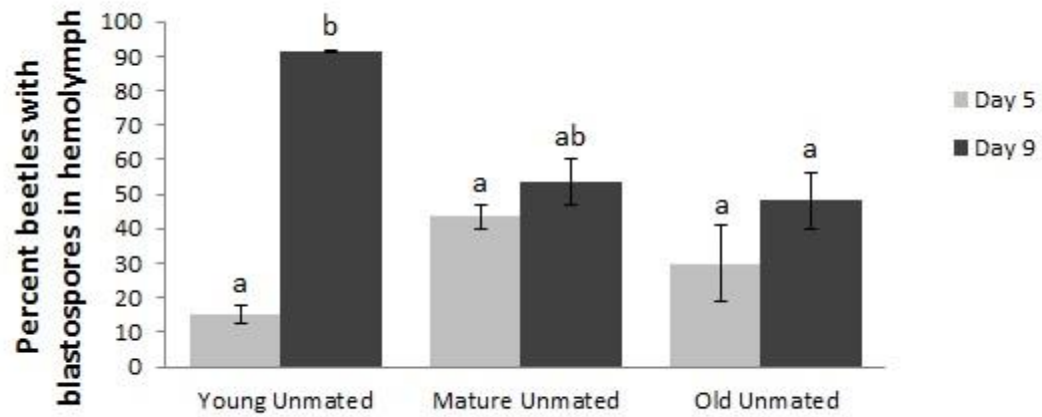


Table 2. The effect of mating and age on the outcome of the infection bioassays and hemolymph assays.

Effect of mating and age	Experiment	Model	Test statistic	DF	P-Value
Mature mated vs. Mature unmated vs. Old mated vs. Old unmated	Days to death	Treatment	$F = 5.17$	3, 3	0.001
		Sex	$F = 17.19$	1, 1	0.0001
	Blastospore presence	Treatment x Day	$\chi^2 = 12.36$	3	0.0062
		Sex	$\chi^2 = 9.66$	1	0.0019
<b>Effect of age</b>					
Young unmated vs. Mature unmated vs. Old unmated	Days to death	Treatment x Sex	$F = 4.65$	2, 124	0.0113
	Blastospore presence	Treatment x Day	$\chi^2 = 16.48$	2	0.0003
		Sex	$\chi^2 = 1.58$	1	0.2085

## 4. Discussion

We hypothesized that there may be a tradeoff between mating and immune competency for *A. glabripennis* and our results suggest that whether there is a cost of mating depends on the age of the adult beetle. Additionally, we hypothesized that adult *A. glabripennis* would undergo immunosenescence and Old beetles would be more susceptible to *M. brunneum* than younger beetles. We found no evidence of immunosenescence in Old beetles as older beetles were not more susceptible to *M. brunneum* than younger beetles.

In this study a cost of mating was only observed for Mature beetles and not for the Old beetles tested (Fig. 8). When inoculated with *M. brunneum*, mated beetles died approximately 1 d faster than unmated beetles. Although Mature beetles died faster when mated compared to Old beetles or unmated Mature beetles there were not more Mature mated beetles that had blastospores in sampled hemolymph on day 5 or day 9 compared to the other treatments (Fig. 10). We hypothesize that mating may make Mature beetles less tolerant (defined as the ability to reduce the impact of a given level of pathogen) of *M. brunneum* infection, although they are not less resistant (defined as the ability to clear pathogens) (Ayres and Schneider, 2012).

Independent of whether or not beetles had mated, Mature and Old females died faster and were more likely to have blastospores in sampled hemolymph than Mature or Old males (Fig. 9, 11). It is possible that reproductively mature females are more susceptible than reproductively mature males because females likely bear a higher cost of reproduction and reproductive behaviors than males in this system. Males most likely have lower energetic costs as they mate and then engage in mate guarding behavior. In



nature, they will fight with other males. However, they readily disperse following an aggressive encounter potentially to limit future aggressive encounters (Keena and Sánchez, 2006), although no male:male encounters occurred during this study. In contrast, female *A. glabripennis* must mate, excavate oviposition pits in the bark of trees and then lay eggs. A female's reproductive behaviors are a continuing cost as females typically lay one egg per day for the majority of their lives (Keena, 2002; Hajek et al. 2008). Even unmated females will chew oviposition pits and oviposit filled unfertilized eggs (unpublished data). In this study females were not given logs in which to oviposit, but they still often chewed pits in twigs and dropped eggs since they couldn't insert them into the twigs.

We did not find evidence of immunosenescence with increasing age for either males or females. These findings suggest that either immunosenescence doesn't occur in these long-lived beetles or we did not test beetles that were old enough to be undergoing immunosenescence. We did not test whether or not beetles were undergoing senescence however, it is very likely the Old females tested in this study were undergoing senescence since healthy female beetles in the lab live an average 10-13 weeks (Keena, 2002) and Old females used in this experiment were an average of 9 weeks old prior to fungal inoculation and then required an average of 1.3-1.6 weeks to die. Additionally, female beetles typically do not lay eggs during the last week of their life, and the viability of eggs tends to drop slightly at the end of the female's lifespan (Keena, 2002). Healthy male beetles in the lab live an average of 14-15 days longer than females, so it is possible that the male beetles used in this experiment were not yet undergoing reproductive senescence. It is likely that longevity of both male and female beetles will be lower in the

field although this has yet to be tested. Beetles in even older age ranges were not tested since too many female beetles would have died before and during the experiment.

The susceptibility of beetles to *M. brunneum* across the three age groups was influenced by sex. Females in all three age groups did not differ in their susceptibility while Young males were more susceptible than older males (Fig. 12). Our evidence suggests that the susceptibility of adult female beetles does not change over the course of the adult life while males become more resistant following sexual maturation. Young (6.5 day-old) male *A. glabripennis* are reproductively immature and cannot successfully mate until they are 11 days old (Keena and Sánchez, 2006) and it is unlikely that 6.5 day-old beetles would have completed boring out of the tree after eclosion (Sánchez and Keena, 2013). Young female beetles were also sexually immature since they require 9-15 days of maturation feeding after eclosion (Keena, 2002; Smith et al., 2002) but it is unknown why they were not more susceptible than older females. It is likely the Young male beetles used in our study were still undergoing sex specific maturation and development and this made them more susceptible to *M. brunneum* than Old males.

We found a cost of mating for Mature females which agrees with the findings of several other studies that also found that reproduction reduced immunocompetence (Fedorka et al., 2004, 2007; Khan et al., 2016; McKean and Nunney, 2001; Rolff and Siva-Jothy, 2002; Short and Lazzaro, 2010; Siva-Jothy et al., 1998). We did not however find a cost of reproduction for Old females and we found that susceptibility to *M. brunneum* changes with age in unmated males. In other systems there is evidence that investments in immunity can change over the course of an invertebrate's lifespan (González-Tokman et al., 2013; Izhar & Ben-Ami, 2015). Maehara and Kanzaki (2014)

also found that newly emerged adult Japanese pine sawyers (*Monochamus alternatus*) were more susceptible than 14 day-old beetles to the fungal pathogen *Beauveria bassiana*, although the authors did not test if this trend was the same for both male and female beetles. Mature male damselflies (*Hetaerina americana*) maintained their reproductive efforts when a nylon thread was implanted while younger males decreased their reproductive efforts when encountering the same challenge; the thread had been inserted to challenge the immune system (González-Tokman et al., 2013).

We found no evidence of a decline in immune competence with increasing age in *A. glabripennis*. Our findings contrast with evolutionary theory and several studies that show evidence of decreased immunocompetence with age (DeVeale et al., 2004; Khan et al., 2016; Maehara and Kanzaki, 2014; Park et al., 2011; Stanley, 2012). However, insects in several other systems also have not shown signs of decreased immunocompetence with age. Old *D. melanogaster* which were undergoing reproductive senescence had enhanced immunity and decreased bacterial loads compared to young flies (Khan and Prasad, 2013). Older leaf-cutting ants, *A. octospinosus*, also did not exhibit immunosenescence and had increased PO activity compared to younger ants (Armitage & Boomsma, 2010). Among honey bees, *A. mellifera*, no evidence of immunosenescence was reported in several studies and instead older bees had enhanced survival upon exposure to the fungal pathogen *M. anisopliae* and the microsporidian pathogen *Nosema ceranae* (Bull et al., 2012; Roberts and Hughes, 2014).

## 5. Conclusions

We found there was a cost of mating to immunity: mated Mature beetles died more quickly than unmated beetles. Contrary to our hypothesis, we found no evidence of immunosenescence in *A. glabripennis*. Young males were more susceptible to *M. brunneum* challenge than Mature or Old males, possibly because they were still undergoing maturation and development. Mature and Old females were more susceptible to *M. brunneum* than males of equal ages and more females had detectable fungal blastospores in their hemolymph compared to Mature and Old males. Based on these findings we hypothesize that in this system females have a higher cost associated with reproduction.

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## Chapter 5. Starvation and imidacloprid exposure influence immune response by *A. glabripennis* to *M. brunneum*

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## Abstract

In several insect systems, fungal entomopathogens have been found to synergize with neonicotinoid insecticides to accelerate host death. Using the Asian longhorned beetle, *Anoplophora glabripennis* [Cerambycidae], an invasive woodborer inadvertently introduced into North America and Europe, we investigated potential mechanisms in the synergy between the entomopathogenic fungus *Metarhizium brunneum* and the insecticide imidacloprid. We hypothesized that a potential mechanism underlying this synergy is imidacloprid's ability to prevent feeding shortly after administration. We investigated whether starvation would have an impact on the mortality of fungal-inoculated beetles similar to pesticide exposure. Using quantitative real-time PCR to quantify fungal load in inoculated beetles we determined how starvation and pesticide exposure impacted beetles' ability to tolerate or resist a fungal infection. We also investigated how starvation and pesticide exposure influence beetle encapsulation and melanization immune responses. We found that starvation had an impact on the survival of *M. brunneum*-inoculated beetles similar to imidacloprid exposure. The synergy, however, is not completely due to starvation since imidacloprid reduced the beetles' melanotic encapsulation response and capsule area while starvation did not significantly reduce these immune responses. Our results suggest that there are multiple interacting mechanisms involved in the synergy between *M. brunneum* and imidacloprid.

## Keywords:

Asian longhorned beetle, *Metarhizium brunneum*, Imidacloprid, Insecticide synergy

# 1. Introduction

Fungal entomopathogens have been found to synergize with neonicotinoid insecticides to increase and accelerate insect mortality (Brito et al., 2008; Doublet et al., 2015; Furlong and Groden, 2001; Jaramillo et al., 2005; Purwar and Sachan, 2006; Russell et al., 2010). However, little research has been conducted to investigate potential mechanisms underlying these synergies and what impact they could have on insect immune responses.

*Anoplophora glabripennis* [Cerambycidae], is an invasive wood borer, native to China and Korea that was inadvertently introduced into North America and Europe (Haack et al., 2010; Hu et al., 2009). The beetle has a wide host range consisting of many species of hardwood trees and is considered an economically important pest of forest and urban trees in North America and Europe (Brabbs et al., 2015; Haack et al., 2010; Hu et al., 2009). Eradication efforts in North America include the use of imidacloprid to protect trees at risk of becoming infested (Haack et al., 2010). The fungal entomopathogen *Metarhizium brunneum* is also being developed for control of *A. glabripennis* (Goble et al., 2016, 2015; Hu et al., 2009). Synergy has been observed in combination treatments, as beetles die significantly faster when exposed to both sublethal doses of imidacloprid and *M. brunneum* than if they are exposed to the fungus alone (Russell et al., 2010).

Imidacloprid is a neonicotinoid insecticide that acts on the insect central nervous system as an agonist of the postsynaptic nicotinic acetylcholine receptors, resulting in continuous stimulation of the neurons which at lethal doses ultimately leads to death (Nauen and Denholm, 2005). At sublethal doses imidacloprid reduces beetle feeding shortly after exposure (Furlong and Groden, 2001; Russell et al., 2010). Starvation was suggested as a potential mechanism for the synergy between imidacloprid and the fungal pathogen *Beauveria bassiana* in Colorado

potato beetles, *Leptinotarsa decemlineata* [Chrysomelidae]. Beetles were inoculated with *B. bassiana* and starved for the same amount of time that they would have decreased their feeding if exposed to imidacloprid alone and it was found that these starved beetles died as quickly as beetles exposed to both imidacloprid and *B. bassiana* (Furlong and Groden, 2001). Feeding by *A. glabripennis*, is reduced for four days following exposure to imidacloprid so starvation may be a potential mechanism for the synergy between imidacloprid and *M. brunneum* in this system (Russell et al., 2010).

We investigated the impact of imidacloprid and starvation on beetles' ability to mount an encapsulation and melanization immune response and resist *M. brunneum*. The ability of insects to mount an effective encapsulation and melanization immune response has been found to be important in defending insects against fungal pathogens (Chouvenc et al., 2009; Gillespie et al., 1997; Hajek and St. Leger, 1994). The encapsulation response involves the formation of an overlapping sheath of hemocytes around a multicellular invader or foreign object and can also involve melanization (Lavine and Strand, 2002). Pesticide exposure has been found to impact insects' encapsulation and melanization immune responses (Dubovskiy et al., 2013) and this may be a potential mechanism for the synergy between an organophosphorus insecticide and the fungal pathogen *M. anisopliae* (Dubovskiy et al., 2010). We also investigated the impact of starvation and pesticide exposure on insects' ability to resist a fungal infection by quantifying the fungal load in insect muscle and hemolymph tissues using real-time PCR. We hypothesize that a potential mechanism for the synergy between *M. brunneum* and imidacloprid is the ability of imidacloprid to prevent feeding shortly after administration. We also hypothesized that pesticide exposure and starvation would make beetles more susceptible to a fungal infection and that

starvation would suppress the encapsulation and melanization immune response against *M. brunneum*.

## 2. Materials and methods

### 2.1 Insect culture

*Anoplophora glabripennis* were reared and tested under quarantine and USDA APHIS permit at Cornell University's Sarkaria Arthropod Research Laboratory. Striped maple (*Acer pensylvanicum*) collected locally was used for adult food and oviposition. Detailed rearing protocols for adults and larvae are described by Goble et al. (2015) and only adults were used in this study. Beetles were maintained at 16:8 h light:dark, with 23 °C days and 16 °C nights, individually in 473 ml lidded clear plastic cups containing striped maple twigs changed weekly. An approximately equal number of male and female beetles were used in each experiment.

### 2.2 Fungal transformation and culture

To avoid false positives due to the presence of naturally occurring *Metarhizium* strains potentially on the field-collected twigs, we avoided the use of endogenous fungal genes as markers for the real-time PCR. Instead, we produced a reporter strain and employed the gene encoding for the Green Fluorescent Protein (GFP) as the target.

#### 2.2.1 Generation of *M. brunneum* F52 strains constitutively expressing *sGFP*

*Metarhizium brunneum* strain F52 (ARSEF 7711; USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY) was transformed to express a Green Fluorescent Protein (GFP). DNA fragments carrying the *bar* resistance cassette, *Aspergillus nidulans olic31* promoter, *sGFP* and *Aspergillus nidulans trpC* terminator were obtained by PCR amplifications using pUCAP NOSII (Donzelli, unpublished), pOHT vector (Yao and Koller, 1995), gGFP (Maor et al., 1998), pOHT vector (Yao and Koller, 1995), respectively as the templates, and the primers listed in Table 3. These fragments were assembled into a pBDU binary vector by USER friendly cloning (Geu-Flores et al., 2007; Donzelli et al., 2012) and transferred into *Agrobacterium tumefaciens* EHA105 by electroporation. *Agrobacterium*-mediated transformation of *M. brunneum* F52 was carried out as described by Moon et al. (2008). Identification of glufosinate ammonium-resistant isolates, which were also fluorescent at 485 nm/528 nm (excitation/emission) wavelengths was followed by the isolation of single conidium progeny. Isolate G15 was used for all the experiments as in preliminary tests it retained a similar level of virulence as the Wild Type (WT).

### 2.2.2 Fungal culture

Fungal cultures were grown for 2-3 weeks in darkness at 26 °C on potato dextrose agar (PDA) (Difco, Sparks, MD) with 0.1 % gentamicin to prevent growth of bacterial contaminants. Suspensions of inoculum were prepared by washing conidia off of cultures by swirling 0.05 % aqueous Tween 80 and 2 mm glass balls to dislodge and suspend conidia. Conidial viability was quantified by suspending conidia in 0.05% aqueous Tween and spreading a 100 µl aliquot of approximately  $1 \times 10^7$  conidia/ml onto each of three 60 mm diam. petri dishes containing PDA. Plates were incubated for 16-17 h at 26 °C, and conidia examined to determine whether conidia



were viable, which was indicated by the growth of germ tubes. Percent conidial viability was determined prior to preparation of conidial suspensions for use in bioassays. Suspensions adjusted to  $10^8$  viable conidia/ml in 0.05 % aqueous Tween using a hemocytometer and used the same day.

*Metarhizium brunneum* was also grown in liquid culture to obtain blastospores (fungal propagules) for generating standard curves for real-time PCR. The blastospore cultures were prepared using a modified procedure described by Ypsilos and Magan (2004) and consisted of deionized (DI) water with 4 % (w/v) cottonseed flour, 4 % glucose and 4 % yeast extract. To prevent the fungi from forming clumps 5 % PEG 200 was added to the culture (Kleespies and Zimmermann, 1992). The culture was simmered for 15 min on a hot plate adjusted to medium heat, strained through two layers of Kimwipes (Kimberly-Clark, Roswell, GA), to remove large particles, and autoclaved. Using a sterile inoculation loop 25 ml of the broth culture was inoculated with *M. brunneum* conidia and placed on an orbital shaker at 175 rpm at 22-23 °C for 3-4 days. The suspension, containing blastospores and mycelia, was strained through two layers of Kimwipes to remove mycelia (as confirmed by microscopy). The suspension was centrifuged for 10 min at 2,500 rpm, the supernatant was discarded, the pellet of blastospores was re-suspended in 50 ml molecular grade water, centrifuged and the supernatant was discarded. The process was repeated 1-2 times using 1.5 ml molecular grade water and centrifugation for 5 min at 14,000 rpm after which the fungal DNA was extracted as described below.

## 2.3 Beetle treatments

Adult *Anoplophora glabripennis* were assigned to one of six treatment groups: Naive, positive controls (fed 1  $\mu$ l of 100 ppm imidacloprid or starved for 66-67 h), exposure to *M. brunneum*, exposure to *M. brunneum* and fed 1  $\mu$ l of 100 ppm imidacloprid, or exposure to *M. brunneum* and starved for 66-67 h.

Beetles were inoculated with *M. brunneum* by shaking single individuals in 11-13 ml of the *M. brunneum*  $10^8$  conidia/ml suspension for 5 sec (solutions were vortexed for 15 sec prior to beetle inoculation and discarded after inoculation). All beetles not in the *M. brunneum* treatments were shaken in 13 ml of sterile 0.05 % aqueous Tween. After treatment, wet beetles were individually placed in plastic cups as described above.

Imidacloprid solutions were prepared by diluting technical grade imidacloprid (Chem Service Inc. West Chester, PA) to 100 ppm in DI water. Beetles were exposed to imidacloprid by placing a 1  $\mu$ l drop of the 100 ppm solution on their mouthparts prior to treatment with either sterile 0.05 % aqueous Tween (imidacloprid alone) or *M. brunneum*. All beetles included in the study fully imbibed the drop. All beetles not in the imidacloprid treatments were fed 1  $\mu$ l of DI water.

Beetles in the starvation treatments were starved for 66-67 h since imidacloprid was found to significantly decrease feeding up to 4 days post-treatment (Russell et al., 2010). After treatment with either sterile 0.05 % aqueous Tween (starvation alone) or *M. brunneum*, beetles were placed in clean 473 ml lidded clear plastic cups that contained only a wet cotton ball for moisture. After 66-67 h beetles were given striped maple twigs that were replaced weekly for the remainder of the experiment.

## 2.4 Bioassays

Beetles in the six treatments were inoculated as described above and maintained under the same rearing conditions as colony beetles. Beetle mortality was monitored daily for 30 d. Cadavers were checked for fungal outgrowth after death. The experiment was repeated three times with a total of 59-60 beetles in each control and *M. brunneum* treatment group. Beetles in the bioassay averaged  $20.3 \pm 0.2$  days old (mean  $\pm$  SE) when treatments began.

### 2.4.1 Data Analysis

Only one control beetle died and it did not exhibit fungal outgrowth. The mortality of fungal-inoculated beetles was analyzed using a proportional hazards model (SAS Institute Inc., 2015). The explanatory variables were replicate, treatment (*M. brunneum* alone, *M. brunneum* x imidacloprid, *M. brunneum* x starvation) and gender. All non-significant interactions were removed from the model. The effect of treatment was analyzed using risk ratios with alpha values adjusted using the Bonferroni correction to take into account multiple comparisons. Median days to death were calculated using Kaplan-Meier survival analyses.

### 2.4.2 Encapsulation and melanization assay

The melanization and encapsulation response of beetles to a foreign object was measured by inserting a 3 mm-long (0.18 mm diam.) nylon filament into the abdomen of beetles 24 h after treatment (see section 2.3). We measured the immune response of beetles to the filaments by quantifying the total darkness of the filament and the area of the capsule that formed around the

filament using a procedure modified from those given by Daukšte et al. (2012) and Rantala et al. (2002). Since encapsulation may or may not include melanization (Lavine and Strand, 2009) and since we cannot distinguish exactly between the deposition of cells compared to the deposition of melanin, as both contribute to the darkness of the filament, we will refer to the total darkness of the filament as “melanotic encapsulation” and the area of the material attached to the filament as “capsule area” (Fig. 14). Prior to insertion, filaments were rubbed with sand paper to facilitate cellular adhesion, autoclaved and then stored in 70% ethanol until use. Before beetles were punctured the insertion site was surface sterilized with 70% ethanol. A small puncture was made between the 2<sup>nd</sup> and 3<sup>rd</sup> abdominal sternites using an insect pin. After the filament was inserted, beetles were returned to cups and maintained either with or without food (starved treatments) for 12 h, after which beetles were cold anesthetized and dissected to retrieve filaments. The assay duration was chosen based on preliminary data that showed 12 h was sufficient to elicit a strong immune response while maintaining differences between individuals so treatment effects were not masked. After removal, filaments were stored individually in 200 µl vials at -20 C until they were photographed. The end of the filament which was deepest within the insect hemocoel was photographed at 100 X on a compound microscope using a Cannon EOS Rebel T1i. Filaments were rotated around the long axis and photographed from three different views. The experiment was repeated five times with a total of 30 beetles per treatment (15 male; 15 female). Beetles used in the encapsulation and melanization assay were on average  $16.4 \pm 0.1$  d old.

#### *2.4.3 Data Analysis*

The melanotic encapsulation response (total gray value) and capsule area (total area of cellular material) on the filaments were analyzed using the image analysis software Fiji

(Shindelin et al., 2012; <http://fiji.sc/>) and the values of the three images collected for each filament were averaged. To control for variation in light levels between photo sessions and to transform the data so high values represented darker filaments, the average gray value of each filament was subtracted from the average of two filaments which had never been inserted into an insect and which were photographed during the same photo session. The encapsulation response was measured as the total area of each filament minus the average area of the two filaments which had never been inserted into an insect.

The mean gray value and mean area of filaments were normally distributed so the data were analyzed using Least Square Means tests and all non-significant interactions were removed from the model. Pair-wise comparisons between treatments were analyzed using Tukey's HSD post-hoc tests (SAS Institute Inc., 2015). Explanatory variables were replicate (random variable), gender, treatment and the interaction between gender and treatment. Separate analyses were conducted to compare the naive to the positive control treatments (naive vs. starved vs. imidacloprid), and the three fungal treatments to each other (*M. brunneum* vs. *M. brunneum* x starved vs. *M. brunneum* x imidacloprid) as well as each fungal treatment and its respective control (*M. brunneum* vs. naive; *M. brunneum* x starved vs. starved; *M. brunneum* x imidacloprid vs. imidacloprid).

## 2.5 Fungal detection using Real-Time PCR assays

### 2.5.1 Tissue collection

Insects were treated as above (section 2.3) and 5 d after treatment hemolymph and muscle tissue samples were collected from beetles in each of the six treatment groups. To collect

hemolymph samples beetles were first cold-anesthetized at 4 °C for 8 min, surface sterilized in 0.825 % sodium hypochlorite, rinsed twice in sterile DI water and blotted dry. Beetles were bled by puncturing the intersegmental membrane between the thorax and abdomen with a sterile insect pin and the hemolymph was collected into a 20 µl microcapillary pipette (Kimble Chase, Vineland, NJ) and placed in 400 µl of lysis buffer AP (DNeasy Plant Mini Kit, Qiagen, Mansfield MA). After bleeding beetles were killed by freezing and immediately dissected to collect approximately 0.04 g of thoracic muscle tissue which was then stored at -20 °C until DNA extraction. The experiment was repeated five times with a total of 41-42 beetles in each fungal treatment and 20-23 beetles in the naive, imidacloprid and starved treatments. Beetles used in the real-time PCR assay were on average  $17.3 \pm 0.2$  d old.

### *2.5.2 DNA extraction*

Fungal DNA was extracted from 20 µl of insect hemolymph, 0.034-0.040 g of insect muscle tissue and pure fungal blastospore cultures using DNeasy Plant Mini Kit (Qiagen, Mansfield MA), according to the manufacturer's directions. Prior to DNA extraction fungal blastospores were lysed with 0.05 g of 0.5 mm diam. zirconia/silica beads (BioSpec Products, Bartlesville, OK) using a Mini-beadbeater (BioSpec Products, Bartlesville, OK) for 90 s at 4200 rpm. DNA was eluted in 65 µL AE buffer and concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham MA).

### *2.5.3 Primers and specificity testing*

A modified real-time PCR protocol developed for measuring the quantity of an EGFP-

transgene (Joshi et al., 2008) was used to determine the quantity of GFP-tagged *M. brunneum* in *A. glabripennis*. The forward primer was modified for use with the GFP protein used in this study and both primers were manufactured by Integrated DNA Technologies (Coralville, IA) while the probe was manufactured by Applied Biosystems (Foster City, CA); forward primer 5'-ccacatgaagcagcagcactt-3', reverse primer 5'-ggtgcgctctggacgta-3' and probe 5'-6FAM-ttcaagtccgcatgcccga-TAMRA-3'.

The specificity of the primers was initially tested by conducting standard PCR assays on DNA extracted from pure culture of *M. brunneum* F52 WT and its GFP-tagged derivative G15, beetles inoculated with F52 WT and naive control beetles. PCR reaction mixtures (25 µl volume) were prepared according to the manufacturer's directions, using the Taq PCR Core Kit (Qiagen, Valencia CA) and contained 0.25 µM of each primer. PCR amplification was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA) programmed for initial denaturation at 95 °C for 5 min; 34 cycles of denaturation at 95 °C for 45 sec, annealing at 60 °C for 1 min; and extension at 72 °C for 5 min. PCR products were visualized in a 1.5 % (wt/vol) agarose gel stained with ethidium bromide. The primers were found to be specific to the GFP-tagged *M. brunneum* G15, as samples without the transgene did not contain the expected 67 bp band.

#### 2.5.4 Real-time PCR protocol

Real-time PCR assays were conducted using the iCycler iQ5 real-time PCR detection system (BioRad Laboratories, Foster, CA). Each reaction mixture (25 µl final volume) contained 2X Universal Probes Master Mix (Bio-Rad), 0.67 µM of each primer, 0.23 µM of probe, and 8 µl of each DNA sample. Assays were run with the following thermal cycling conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and

single-step annealing and extension at 60 °C for 1 min. Independent triplicates of each sample were run. Each PCR assay included a standard curve (a series of five 10-fold dilutions, from 2 ng to 0.2 pg total DNA from *M. brunneum* strain G15) for determining starting DNA concentration of hemolymph or muscle samples (based on  $C_t$  values at 90-101% assay efficiency), a non-template control and two control samples from the naive, imidacloprid and starved treatments (either a hemolymph and muscle tissue sample from each treatment or two hemolymph samples from each treatment) and the rest of the plate was used to test hemolymph and muscle tissue samples from *M. brunneum*-inoculated beetles. Only a subset of the muscle tissue samples were run (13-14 from each fungal treatment and 9-10 from each control treatment), since early data demonstrated no significant differences in the amount of fungi present in hemolymph compared to muscle tissue samples ( $F_{1, 67.99} = 0.2068$ ,  $P = 0.6507$ ). The limit of detection for this assay was determined to be 0.1 pg of *M. brunneum* DNA.

#### 2.5.5 Data analysis

The ratio of fungal to total DNA (insect + fungal DNA) in each sample was obtained by averaging the amount of fungal DNA detected in each sample replicate, divided by the total volume of the DNA in the sample (8  $\mu$ l) and then dividing by the concentration of total DNA in the insect sample as obtained by NanoDrop. The data were normalized using a  $\log(x + 1 \times 10^{-6})$  transformation, analyzed using Least Square Means tests and all non-significant interactions were removed from the model (SAS Institute Inc., 2015). The explanatory variables were experimental replicate (random variable), individual beetle (random variable), gender, treatment, tissue type (hemolymph or muscle), and the interaction between gender and treatment. The presence or absence of GFP-tagged *M. brunneum* in the hemolymph and muscle tissue of



inoculated beetles was determined using a logistic regression for which the explanatory variables were experimental replicate (random variable), individual beetle (random variable), gender, tissue type and treatment (R Development Core Team, 2016). A Likelihood Ratio test which excluded the factor of interest was used to test treatment effects.

### 3. Results

#### 3.1 Bioassay

Beetles that were inoculated with *M. brunneum* and either starved or exposed to imidacloprid died 6 d faster than those exposed to *M. brunneum* alone [ $\chi^2_2 = 39.96$ ,  $p < 0.0001$ ; risk ratios,  $p \leq 0.0167$ ; median days to death: *M. brunneum*, 17 d (CI: 14, 18); imidacloprid x *M. brunneum*, 11 d (CI: 10, 12); starvation x *M. brunneum* 11 d (CI: 10, 12)] (Fig. 15). Across all treatments, females were significantly more susceptible to *M. brunneum* and died 2 d faster than males [ $\chi^2_1 = 11.32$ ,  $p = 0.0008$ ; median days to death: Females, 11 d (CI: 10, 13); Males, 13 d (CI: 12, 14)] (Fig. 16). The interaction between treatment and gender was not significant, so it was removed from the model.

#### 3.2 Melanization and encapsulation assay

Exposure to the fungal pathogen alone significantly increased the melanotic encapsulation response compared to naive controls while the area of the capsule was close to being significantly different than naive controls (melanotic encapsulation,  $F_{1, 53.07} = 53.02$ ,  $P = 0.0131$ ; capsule area,  $F_{1, 53.42} = 3.32$ ,  $P = 0.0739$ ) (Fig. 17, Table 4). Exposure to imidacloprid or

starvation however depressed encapsulation and melanization responses upon fungal exposure and beetles in these treatments did not have significantly different melanotic encapsulation rates or capsule areas when compared with their respective controls (*M. brunneum* x imidacloprid vs. imidacloprid: melanotic encapsulation,  $F_{1, 51.09} = 0.03$ ,  $P = 0.8575$ ; capsule area,  $F_{1, 51.11} = 0.80$ ,  $P = 0.3750$ ; *M. brunneum* x starved vs. starved; melanotic encapsulation,  $F_{1, 50.52} = 0.52$ ,  $P = 0.0725$ ; capsule area,  $F_{1, 50.24} = 3.37$ ,  $P = 0.0725$ ) (Table 4). Among fungal-inoculated beetles, exposure to imidacloprid reduced both capsule area and melanotic encapsulation while starvation did not significantly affect either melanotic encapsulation or the area of capsules (melanotic encapsulation,  $F_{2, 80.09} = 3.48$ ,  $P = 0.0355$ ; capsule area,  $F_{2, 80.25} = 3.45$ ,  $P = 0.0365$ , Tukey's HSD,  $p \leq 0.05$ ) (Figs. 18, 19, Table 4). There was no significant effect of treatment on capsule area or the melanotic encapsulation response of beetles in the naive, imidacloprid or starved treatments (melanotic encapsulation,  $F_{2, 78.99} = 0.21$ ,  $P = 0.8151$ ; capsule area,  $F_{2, 79.23} = 1.18$ ,  $P = 0.3125$ ) (Table 4). All interactions for all models were not significant and were therefore removed. Additionally there was no effect of gender for any of the models (Table 4).

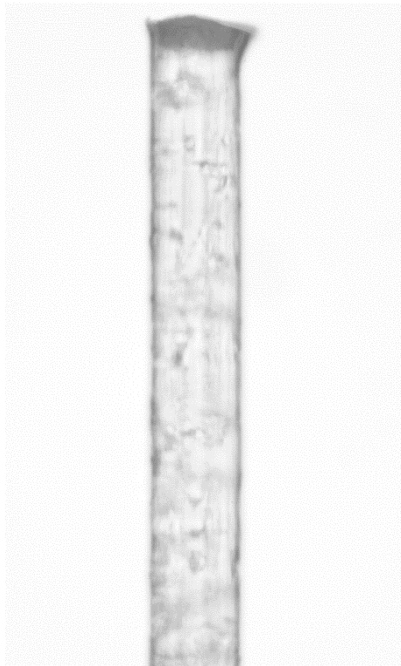
### 3.3 Real-time PCR Assays

All controls were found to be negative for fungal DNA. There was no effect of gender or tissue type on the amount fungi present in naive, imidacloprid, starved or fungal-inoculated insect tissues (Gender,  $F_{1, 157.2} = 0.24$ ,  $P = 0.6285$ ; Tissue type,  $F_{1, 67.99} = 0.21$ ,  $P = 0.6507$ ). Among fungal-inoculated beetles there was no effect of treatment and exposure to imidacloprid or starvation did not increase the fungal load ( $F_{2, 105.1} = 0.66$ ,  $P = 0.5890$ ) (Fig. 20). Additionally, the levels of fungi detected in hemolymph compared to muscle tissue were not significantly different ( $F_{1, 45.36} = 0.10$ ,  $P = 0.7583$ ). There was no effect of gender and female beetles were not

more likely to have fungi in their hemolymph compared to males ( $F_{1, 101.5} = 0.58$ ,  $P = 0.4462$ ).

All interactions were not significant and were therefore removed from the model. Among fungal-inoculated beetles there was also no effect of treatment, tissue type or gender on whether or not fungi were present or absent (Treatment,  $\chi^2_2 = 2.12$ ,  $p = 0.3469$ ; Tissue type,  $\chi^2_1 = 2.34$ ,  $p = 0.1261$ ; Gender,  $\chi^2_1 = 1.46$ ,  $p = 0.227$ ).

Figure 14. Nylon filaments photographed at 100 X. A. Control filament which was never inserted into an insect. B. Filament inserted into beetle for 12 h with melanotic encapsulation response



**A. Control**



**B. Inserted into  
beetle for 12 h**

Figure 15. Percentages of positive control (naive, imidacloprid and starved combined) beetles and *M. brunneum*-inoculated beetles surviving over time. Different letters indicate significant differences in survival curves between treatments (Tukey's HSD,  $p \leq 0.05$ ).

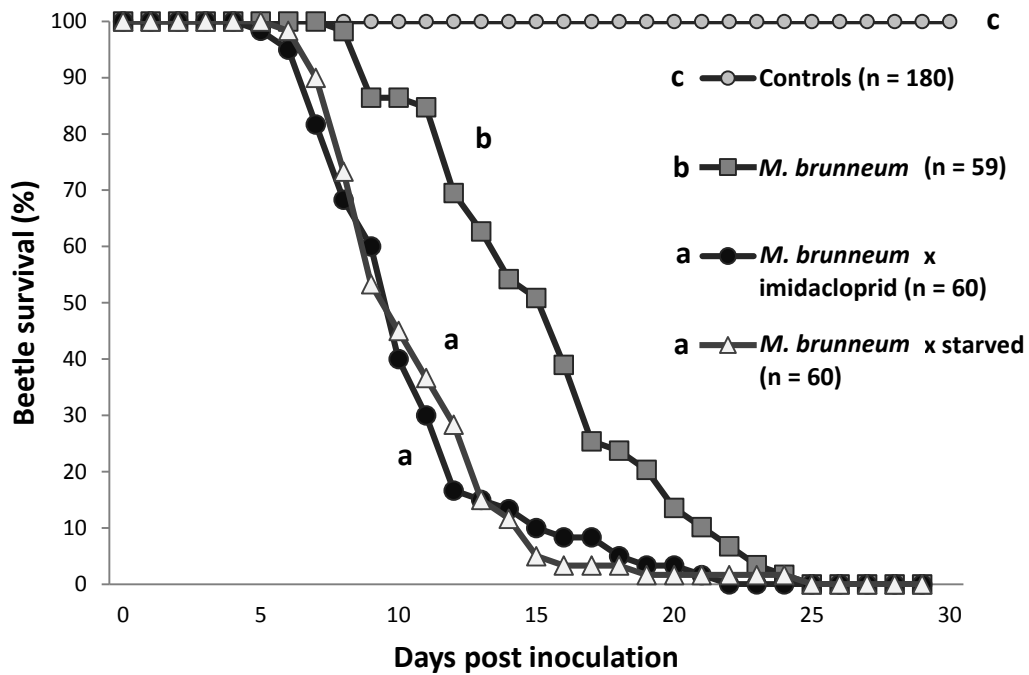


Figure 16. Percentages of beetles (treatments combined) inoculated with *M. brunneum* surviving over time. Different letters indicate significant differences in survival curves between genders across all treatments (Tukey's HSD,  $p \leq 0.05$ ).

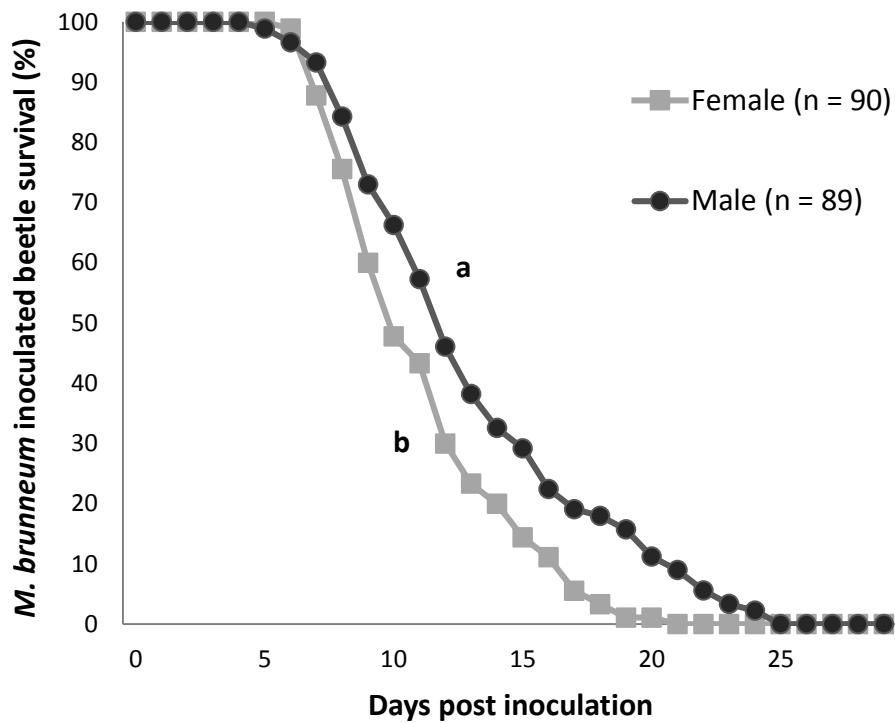


Figure 17. Melanotic encapsulation response of naive vs. *M. brunneum*-inoculated beetles, higher values are darker. Different letters indicate significant differences between treatments ( $F_{1, 53.07} = 53.02$ ,  $P = 0.013$ ).

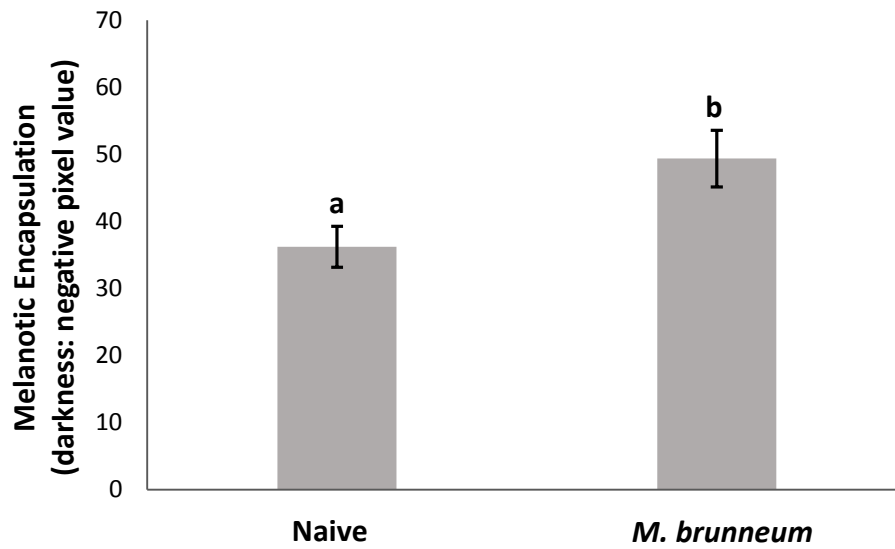


Figure 18. Melanotic encapsulation response of *M. brunneum*-inoculated beetles, higher values are darker. Different letters indicate significant differences between treatments (Tukey's HSD,  $p \leq 0.05$ ).

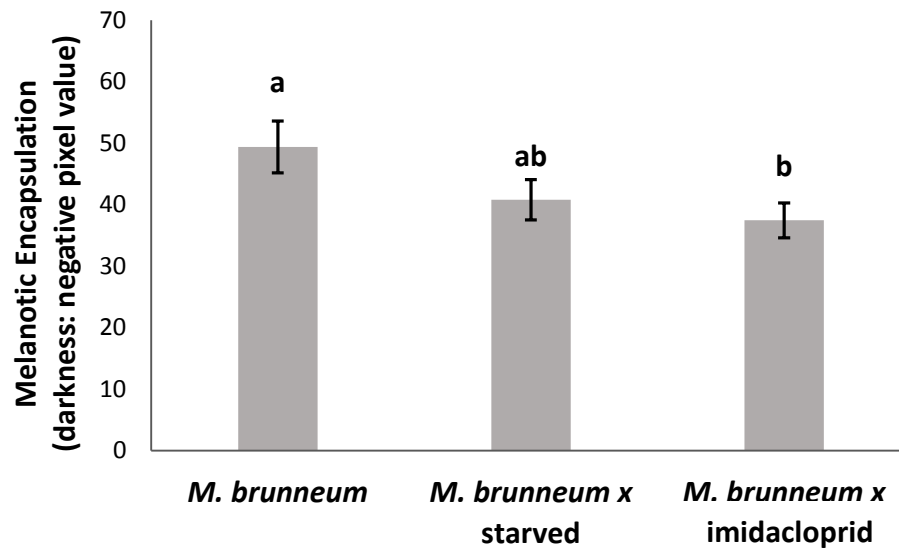




Figure 19. Capsule area of *M. brunneum*-inoculated beetles. Different letters indicate significant differences between treatments (Tukey's HSD,  $p \leq 0.05$ ).

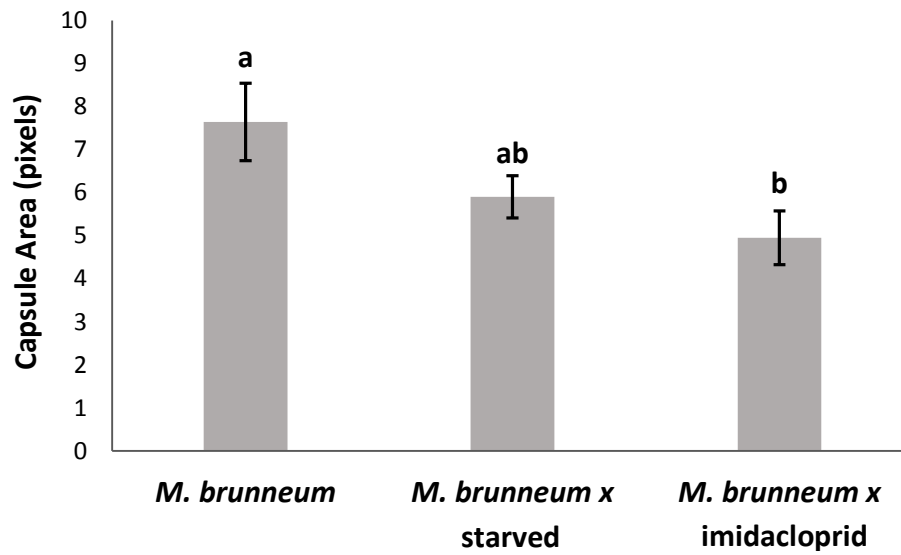


Figure 20. Ratio of fungal DNA (ng) to total DNA (ng) present in *M. brunneum*-inoculated beetles ( $F_{2, 105.1} = 0.66$ ,  $P = 0.589$ ).

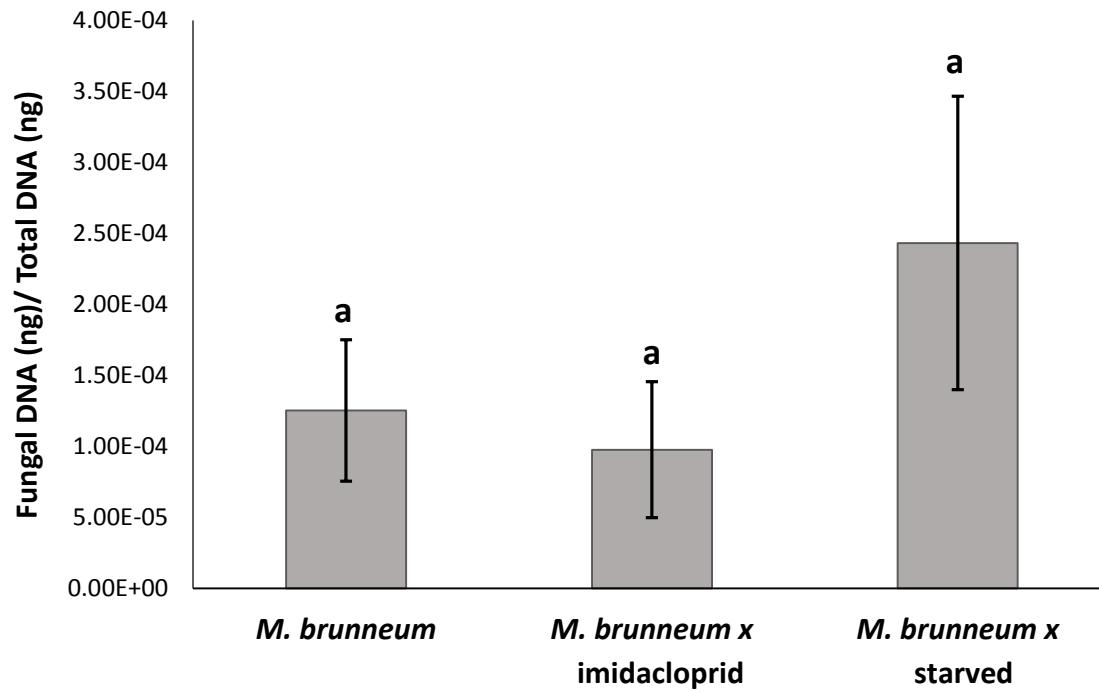


Table 3. Primers used for the construction of the sGFP-tagged *M. brunneum* F52 G15 strain.

Primer	Sequence 5'=>3'	Remarks
oliC-UF	AGGATGAdUGCCTGCAGCTGTGGAGCC	amplification of <i>A. nidulans olic31</i> promoter
oliC-UR	ACGTCAAGdUGTGATGTGATGGAGTTGAG	amplification of <i>A. nidulans olic31</i> promoter
sGFP-UF:	ACTTGACGdUatgGTGAGCAAGGGCG	amplification of <i>sGFP</i> coding region
sGFP-UR:	AGTGGATGdUTTACTTGTACAGCTCGT	amplification of <i>sGFP</i> coding region
Ttrpc-UF	ACATCCACdUACTTAACGTTACTGAAATC	amplification of <i>A. nidulans trpc</i> terminator
Ttrpc-UR:	GGAGACAdUCTAGAAAGAAGGATTACC	amplification of <i>A. nidulans trpc</i> terminator
BarExpr-2F	GGGAAAGdUAGAAGATGATATTGAAGGA	<i>bar</i> resistance cassette
BarExpr-2R	ATCATCCdUC TTTGACAGCTTATCAT	<i>bar</i> resistance cassette

dU = deoxyuridine

Table 4. Melanization and encapsulation assay results

Treatments	Measurement	Model	F-Statistic	DF	P-Value
Naive vs. Starved vs. Imidacloprid	Melanotic encapsulation	Treatment	0.21	2, 78.99	0.8151
		Gender	0.00	1, 80.48	0.9985
	Capsule Area	Treatment	1.18	2, 79.23	0.3125
		Gender	0.02	1, 82.58	0.8847
<i>M. brunneum</i> vs. Naive	Melanotic encapsulation	Treatment	53.02	1, 53.02	0.0131
		Gender	0.05	1, 54.91	0.8233
	Capsule Area	Treatment	3.32	1, 53.42	0.0739
		Gender	0.00	1, 56.93	0.9919
<i>M. brunneum</i> x starved vs. Starved	Melanotic encapsulation	Treatment	0.52	1, 50.52	0.0725
		Gender	0.23	1, 51.54	0.6352
	Capsule Area	Treatment	3.37	1, 50.24	0.0725
		Gender	0.01	1, 52.06	0.9389
<i>M. brunneum</i> x imidacloprid vs. Imidacloprid	Melanotic encapsulation	Treatment	0.03	1, 51.09	0.8575
		Gender	2.45	1, 52.29	0.1233
	Capsule Area	Treatment	0.80	1, 51.11	0.3750
		Gender	0.57	1, 54.12	0.4546
<i>M. brunneum</i> vs. <i>M. brunneum</i> x starved vs. <i>M.</i> <i>brunneum</i> x imidacloprid	Melanotic encapsulation	Treatment	3.48	2, 80.09	0.0355
		Gender	0.06	1, 81.13	0.8102
	Capsule Area	Treatment	3.45	2, 80.25	0.0365
		Gender	0.13	1, 82.69	0.7146

## 4. Discussion

We hypothesized that a potential mechanism of the synergy between *M. brunneum* and imidacloprid is imidacloprid's ability to prevent feeding shortly after administration (Furlong and Groden, 2001; Russell et al., 2010). We found that starvation plus *M. brunneum* exposure had a similar impact on beetle mortality as exposure to *M. brunneum* plus a sublethal dose of imidacloprid, and beetles receiving either of these treatments died 6 d faster than beetles exposed to *M. brunneum* alone. We also hypothesized that starvation and exposure to imidacloprid would reduce beetles' ability to mount an immune response against a foreign object. We found that exposure to imidacloprid prevented an increase compared to *M. brunneum* inoculation alone in the melanization and encapsulation responses. Although starved beetles died as quickly as beetles exposed to both imidacloprid and *M. brunneum*, the ability of imidacloprid to suppress the encapsulation and melanization response is due to more than its ability to prevent feeding since the melanization and encapsulation responses of starved beetles were not significantly reduced compared to *M. brunneum*-inoculated beetles. We also hypothesized that pesticide exposure and starvation would make beetles less resistant to a fungal infection. However, we found that these beetles didn't have higher fungal loads, suggesting starvation and pesticide exposure made them less tolerant, but not less resistant to *M. brunneum*.

We found that starvation and *M. brunneum* exposure had a similar impact on beetle mortality as beetles exposed to a sublethal dose of imidacloprid and *M. brunneum*; beetles with both treatments died 6 d faster than beetles exposed to *M. brunneum* alone. Our results agree with those of Furlong and Groden (2001), who found that starvation for 24 h and exposure to *B. bassiana* had a similar synergistic effect on beetle mortality as exposure to imidacloprid and *B. bassiana*.

Exposure to *M. brunneum* enhanced beetles' encapsulation response 36 h after fungal infection, which is early in the infection process but after fungal penetration has begun (Chouvenc et al., 2009). An increase in the encapsulation response following fungal infection has also been found in *Apriona germari* [Cerambycidae] beetles infected with *B. bassiana* (Li et al., 2009) and *Reticulitermes flavipes* termites infected with *M. anisopliae* (Chouvenc et al., 2009). A decline in the melanization and encapsulation response however was found in *L. decemlineata* larvae infected with *M. anisopliae* compared to controls two days post-infection (Dubovskiy et al., 2010). However this is likely because the encapsulation response had begun to decline following infection since the peak encapsulation response in *A. germari* beetles infected with *B. bassiana* occurred 2.5 d after invasion of the fungus (Li et al., 2009). It is possible that we would have seen a similar result in our study if the filaments were inserted later in the fungal infection process.

Upon fungal infection, imidacloprid prevented an increase in the melanization and encapsulation responses which were significantly reduced compared to beetles exposed only to *M. brunneum*. These findings suggest that suppression of the melanization and encapsulation responses may be a possible mechanism for the synergy between *M. brunneum* and imidacloprid. Imidacloprid alone did not suppress the encapsulation and melanization responses below those seen in control naive or starved insects; rather it prevented the increase of this immune response upon fungal infection.

The ability of imidacloprid to suppress these cellular and humoral immune defenses is due to more than its ability to prevent feeding since the melanization and encapsulation responses of starved beetles were not significantly reduced compared to *M. brunneum*-inoculated beetles. In *Tenebrio molitor* starvation had no impact on beetles' encapsulation responses

although reduced phenoloxidase (PO) activity was reported (Siva-Jothy and Thompson, 2002). Since we only measured the encapsulation and melanization response of the insects it is likely that starvation may impact another aspect of the immune response that we did not measure. Pesticides have been found to impact multiple aspects of insects' humoral and cellular immune responses including PO, melanization and encapsulation (reviewed in James and Xu, 2012). Less is known about how neonicotinoid insecticides impact insect immunity but it has been found that dual exposure to imidacloprid and the microsporidian fungal pathogen *Nosema ceranae* repressed the expression of immunity-related genes including those involved in midgut immunity and trehalose metabolism (Aufauvre et al., 2014). In honey bees, *Apis mellifera*, imidacloprid and the neonicotinoid clothianidin were found to negatively affect NF- $\kappa$ B signaling and reduce expression of the antifungal peptide *drosomycin* (Prisco et al., 2013), which has also been implicated in antifungal immune defense in *Drosophila melanogaster* (Zhang and Zhu, 2009).

Even though beetles exposed to starvation or imidacloprid were more susceptible to *M. brunneum*, they did not have higher fungal loads, which suggests they are less tolerant (defined as the ability to reduce the impact of a given level of pathogen) of *M. brunneum* infection, although they are not less resistant (defined as the ability to clear pathogens) (Ayres and Schneider, 2012). Our findings contrast with those of Prisco et al. (2013), who found that imidacloprid led to increased replication of a viral pathogen. However, our results are consistent with those of a previous study we conducted (Fisher and Hajek, 2016) in which we found that more susceptible insects were not more likely to have detectable levels of fungal propagules in their hemolymph compared to less susceptible individuals. It is also possible that we would have detected differences in infection levels of more susceptible compared to less susceptible insects if tissues were sampled later in the infection process as our previous study showed that the

presence of blastospores in the hemolymph of beetles increased from day 5 to day 9 (Fisher and Hajek, 2016). We chose to sample tissues at day 5 since it is prior to when *A. glabripennis* generally begin dying from inoculation with this dose of *M. brunneum* F52 (Fisher and Hajek, 2016). Although 41.9 % of fungal-inoculated beetles in this experiment did not have detectable levels of fungi in hemolymph, it is very likely these beetles were infected but the fungus was below detectable levels on day 5, as all fungal-inoculated beetles in the bioassay experiment died and all but one beetle sporulated. It is likely that fungi would have been detected in more of the beetles if they were sampled at a later time following fungal inoculation.

Another potential mechanism explaining the synergy between neonicotinoid insecticides and fungal pathogens could be the ability of these insecticides to inhibit behavioral defenses (reviewed in James and Xu, 2012) such as allo-grooming in ants (Galvanho et al., 2013). In *A. glabripennis* it is unlikely that the increased susceptibility of *A. glabripennis* to *M. brunneum* upon pesticide exposure is due to an inhibition of grooming behaviors since *A. glabripennis* do not increase their grooming behaviors upon fungal infection (Fisher, unpublished data).

Our results show that the synergistic effect of the fungal pathogen *M. brunneum* and the pesticide imidacloprid on *A. glabripennis* mortality is partly, but not solely, due to a reduction in feeding upon pesticide exposure. Imidacloprid prevented an increase in the encapsulation and melanization response of *M. brunneum*-inoculated beetles and reduced beetles' tolerance but not resistance to the pathogen. Our findings have important implications for pest management as the use of microbial control organisms is increasing due to an increase in organic agriculture (Economic Research Service USDA, 2015) and pressure on growers, particularly in Europe, to adopt Integrated Pest Management strategies to reduce their pesticide use (Lacey et al., 2015). Moreover, microbial control organisms that synergize with pesticides can create opportunities for



pesticides to be applied at lower rates and can be used to help delay the onset of insecticide resistance (Ambethgar, 2009). Additionally, findings that at sublethal doses pesticides can negatively impact beneficial arthropods and increase the susceptibility of bees to pathogens highlights the importance of studying the impacts of sublethal pesticide exposure on beneficial arthropods (Desneux et al., 2007; Pettis et al., 2013). Therefore it is critical to understand how pesticides and pathogens interact to affect insect immunity.

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## Chapter 6. General conclusions

I have presented my studies of the ecoimmunology of the invasive Asian longhorned beetle, *Anoplophora glabripennis*. Specifically, I investigated how the behavior, life history and age of these beetles affect their ability to combat a pathogen challenge. I also investigated how pesticide exposure, high temperatures and prior maternal pathogen exposure influence the immune response and susceptibility of these beetles to the fungal pathogen *Metarhizium brunneum* which is being developed for their biological control. These findings broaden our knowledge of ecoimmunology in beetles and provide insight into insect-pathogen interactions.

My findings also have implications for the biological control of *A. glabripennis* which is an invasive wood-boring pest. For example, exposure of beetles to high temperatures increased their survival against a fungal pathogen (Chapter 2). However, when given the choice, fungal-exposed beetles did not exhibit behavioral fever by elevating their preferred body temperature. Evidence was also found that beetles do not exhibit basking behaviors in the laboratory and therefore may not be physiologically adapted to exhibit behavioral fever upon pathogen exposure. These findings suggest that Asian longhorned beetles may not exhibit behaviors that reduce their pathogen load and the efficacy of biological control using *M. brunneum*.

Relatively little is known about how prior parental pathogen exposure can influence offspring immunity in insects and specifically whether or not transgenerational immune priming (TGIP) can occur in response to fungal pathogen exposure. Through my research I found evidence for TGIP in *A. glabripennis* since maternal exposure to a gram negative bacterium as well as a fungal pathogen increased offspring survival (Chapter 3). I found that whether or not

mothers, which were exposed to *M. brunneum*, conveyed enhanced protection to their offspring was dependent on whether or not the pathogen was alive or dead. This finding provided the first evidence that the viability of a pathogen (whether it is alive or dead) can influence whether or not TGIP occurs in insects. Even though maternal exposure to live *M. brunneum* enhanced offspring survival, the survival of offspring was increased by only 3.5 days, which is unlikely to impact fungal biocontrol in the field because these beetles have a long adult lifespan (Keena, 2002; Smith et al., 2002).

Mating and insect age both influence insect immunity (Chapter 3). There was only a cost of mating for mature but not old beetles which demonstrates that the susceptibility of beetles is influenced by both age and reproductive status. The old beetles used in this study were not more susceptible to *M. brunneum* than Mature and Young beetles. These findings contradict evolutionary theory which postulates that as organisms age, their immunocompetence will decline (Stanley, 2012). In the present study, young male *A. glabripennis* were more susceptible to pathogens than older males. Sexual maturation may influence insect immunity in a sex-specific manner and future studies should be conducted to explore this topic.

The pesticide imidacloprid is used to control *A. glabripennis* in the field (Haack et al., 2010) and sub-lethal doses of imidacloprid have been found to synergize with *M. brunneum* (Russell et al., 2010). I found that starvation, which is induced upon exposure to imidacloprid, is a likely mechanism of the synergy between imidacloprid and *M. brunneum* (Chapter 5). Exposure of beetles to imidacloprid also inhibited their ability to mount melanization and encapsulation immune responses upon exposure to *M. brunneum*, thus increasing their susceptibility to *M. brunneum*. My findings that pesticides can influence insect immunity have important implications for pest management as well as conservation biology (Chapter 5).

Additionally since the immunocompetence of beneficial arthropods, particularly pollinators, can be compromised by exposure to sub-lethal doses of pesticides (Desneux et al., 2007; Pettis et al., 2013) these findings provide additional insights into how pesticides and pathogens can interact to reduce host immunocompetence. In summary, *A. glabripennis* age, mating status, prior maternal pathogen exposure and exposure to a pesticide all influence *A. glabripennis* immunity upon exposure to the fungal pathogen *M. brunneum*.

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## Appendix 1. Supplementary data for chapter 3

Table S1. Sample sizes for offspring bioassays. Sample sizes for offspring from the maternal treatment and control groups inoculated with *M. brunneum* and untreated offspring used as negative controls.

Pathogen Treatments <sup>1</sup>	Rep	Total
Heat-killed <i>S. marcescens</i>	1	21
	2	29
	3	12
Total		62
Heat-killed <i>M. brunneum</i>	1	33
	2	29
	3	25
Total		87
Living <i>M. brunneum</i>	1	37
	2	19
	3	27
Total		83
Living <i>M. anisopliae</i>	1	16
	2	26
	3	9
Total		51
Control Treatments	Rep	Total
Naive	1	19
	2	21
	3	20
Total		60
Broth injection	1	4
	2	12
	3	11
Total		27
GIM <sup>2</sup> injection	1	14
	2	19
	3	19
Total		52
Untreated <sup>3</sup>	1	19
	2	21
	3	20
Total		60

<sup>1</sup>Corresponding controls listed in Table 1

<sup>2</sup>Grace's Insect media

<sup>3</sup>Untreated beetles were not exposed to *M. brunneum*

## Figure Legends

Figure S1. Survival curves for offspring of the heat-killed *M. brunneum* treatment. Percentages of male and female (merged) offspring treated with *M. brunneum* surviving over time whose mothers were challenged with either heat-killed *M. brunneum* or a control treatment (naive control or GIM injection). There were no significant differences between treatment survival curves ( $\chi^2_2 = 0.74$ ,  $p = 0.6896$ ).

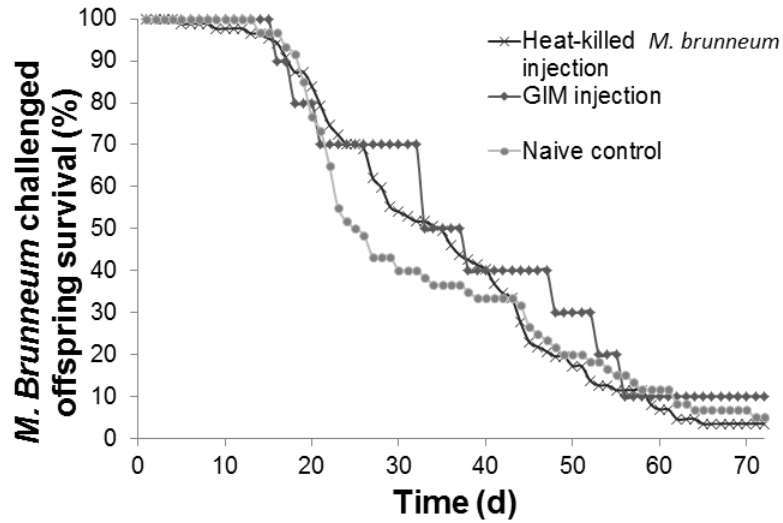
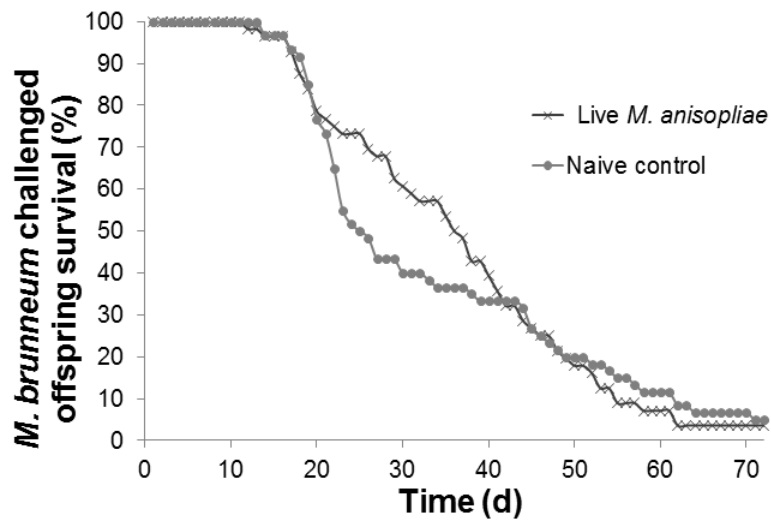


Figure S2. Survival curves for offspring of the live *M. anisopliae* treatment. Percentages of male and female (merged) offspring treated with *M. brunneum* surviving over time whose mothers were challenged with either a living dose of *M. anisopliae* or a control treatment (naive control). There were no significant differences between treatment survival curves ( $\chi^2_1 = 0.13$ ,  $p = 0.7216$ ).





## Appendix 2. Supplementary data for chapter 4

Figure S1. Median days to death  $\pm$  95% CI of Old control and fungal-inoculated female beetles.

Different letters signify significant differences between treatments ( $F_{1,1} = 11.80$ ,  $P = 0.0006$ ).

